Funktionelle Untersuchungen der beiden Alien-Isoformen Alienα und CSN2

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1. Einleitung

Die meisten Proteine agieren nicht alleine, sondern sind in komplexe Netzwerke eingebunden. Erst durch die Interaktion mit anderen Proteinen können sie dann ihre Funktion erfüllen. Aus diesem Grund kann anhand der Bindungspartner auf die physiologischen Funktionen eines Proteins geschlossen werden [Coulombe *et al.*, 2004]. Dabei besitzt ein großer Teil der Proteine nur eine begrenzte Anzahl an Bindungspartnern und damit nur wenige, distinkte biologische Funktionen. Diese Proteine sind meist fest in einen Komplex eingebunden und sind nur in diesem Kontext aktiv [Peth *et al.*, 2007]. Dagegen existieren einige Proteine, wie p53, BubR1 oder c-Myc, die in verschiedenste Signalwege integriert sind. Dadurch wird gewährleistet, dass vielfältige Informationen über den Zustand der Zelle integriert werden können und eine umfassende Antwort eingeleitet werden kann [Mao *et al.*, 2003; Kaustov *et al.*, 2006; Kuttler & Mai, 2006].

Alien wurde mit vielen verschiedenen physiologischen Funktionen in Verbindung gebracht [Tenbaum *et al.*, 2007; Leal *et al.*, 2008]. Deshalb stellt Alien potentiell auch einen solchen multifunktionalen Regulator dar. Bisher konnte sein Interaktions- und damit sein Funktionsspektrum nur unzureichend beschrieben werden. Somit ist eine weitere Untersuchung dieses Proteins für dessen umfassende Charakterisierung essentiell.

1.1 Alien

Alien wurde in den letzten Jahren als Faktor beschrieben, der zusammen mit seinen Interaktionspartnern sowohl die Transkription, die posttranslationalen Modifikationen als auch den Abbau verschiedener Proteine beeinflusst [Naumann *et al.*, 1999; Lyapina *et al.*, 2001; Tenbaum *et al.*, 2003; Huang *et al.*, 2005]. Bisher wurden zwei Isoformen von Alien beschrieben: Alienα und Alienβ. In der Literatur werden verschiedene Bezeichnungen verwendet – unter anderem *COP9 signalosome complex subunit 2* (COPS2), *Signalosome subunit 2* (CSN2) oder *Thyroid hormone receptor interactor 15* (TRIP15) – die nicht klar zwischen den Isoformen unterscheiden. Zur eindeutigen Benennung wird in der vorliegenden Arbeit Alien als Oberbegriff für beide Isoformen verwendet. Alienα bezeichnet die kleinere Variante, während CSN2 die größere Isoform darstellt. Beide Subtypen sind hoch konserviert, wie sich an der 90%igen Identität der Aminosäuresequenz zwischen Mensch und *Drosophila* zeigt [Dressel *et al.*, 1999]. Das circa 51,6 kDa schwere CSN2 und das rund 36 kDa große Alienα entstehen aus derselben circa 2000 Basen umfassenden mRNA. Durch einen bisher unbekannten Mechanismus, der wahrscheinlich auf posttranslationalem Editing beruht, fehlt

dem 305 Aminosäuren langen Alienα die C-terminale Domäne des vollständigen Proteins, das aus 443 Aminosäuren besteht [Dressel *et al.*, 1999]. Der C-Terminus beinhaltet die PCI-Domäne. Diese Domäne scheint essentiell für die Assemblierung des COP9-Signalosoms, obwohl ihre exakte Funktion bei diesem Prozess noch nicht aufgeklärt werden konnte [Lykke-Andersen *et al.*, 2003; Huang *et al.*, 2005]. Darüber hinaus besitzen beide Alien-Isoformen eine saure Region im N-Terminus und ein mögliches Zink-Finger-Motiv im C-terminalen Teil. In der Core-Domäne befindet sich eine hydrophobe Region, die von zwei potentiellen α-helikalen Strukturen und einer möglichen Kernlokalisationssequenz flankiert wird [Dressel *et al.*, 1999].

Alien ist ein Mitglied der nukleären Hormonrezeptor-Superfamilie. Dabei wirkt es als Corepressor des Thyroidhormon-Rezeptors, wenn dieser kein Hormon gebunden hat [Dressel *et al.*, 1999; Burke & Baniahmad, 2000]. Dabei konnten zwei Repressor-Domänen – eine am N-Terminus (Aminosäure 1-128) und eine zweite am C-Terminus (Aminosäuren 266-305) – lokalisiert werden [Moehren *et al.*, 2004]. Die reprimierende Wirkung wird wahrscheinlich von dem Alien-Interaktionspartner Sin3a vermittelt, das Histondeacetylasen und Histonmethyltransferasen rekrutiert. Die dadurch hervorgerufene Kondensierung des Chromatins führt zu einer Stilllegung der darin organisierten Gene [Moehren *et al.*, 2004]. Bindet dagegen das Thyroidhormon an den Rezeptor, diffundiert Alien von diesem ab. Dadurch kann der Thyroidhormon-Rezeptor mit Coaktivatoren assoziieren, die die Anschaltung der Transkription bestimmter Gene bewirken [Lee et al., 1995]. Des Weiteren wurden die Interaktionen mit dem nukleären Hormonrezeptoren DAX-1 und dem Vitamin-D-Rezeptor beschrieben, eine Bindung an die Retinsäurerezeptoren RAR und RXR konnte aber bisher nicht nachgewiesen werden [Altincicek *et al.*, 2000; Polly *et al.*, 2000].

In Mäusen konnte die Expression von Alien in vielen verschiedenen Geweben, wie zum Beispiel Leber, Niere, Brustdrüse, Lymphknoten, Plazenta und Gehirn nachgewiesen werden. Der Corepressor scheint bei der Entwicklung des Gehirns eine entscheidende Rolle zu spielen. Wird das Gen, das für CSN2 kodiert, bei Mäuseembryonen ausgeschaltet, sterben diese bereits kurz vor der Einnistung in den Uterus ab [Lykke-Andersen *et al.*, 2003]. Diese Daten belegen, dass Alien eine wichtige Stellung in der Zelle einnimmt und essentiell für deren Viabilität ist.

1.2 Der COP9-Komplex

CSN2 ist ein wesentlicher Bestandteil des COP9-Komplexes. Dieser Komplex wurde ursprünglich als negativer Regulator der Photomorphogenese in *Arabidopsis* beschrieben [Wei *et al.*, 1994; Wei & Deng, 1999]. Später wurde nachgewiesen, dass der COP9-Komplex, der auch als Signalosom bezeichnet wird, in Organismen von *Schizosaccharomyces pombe* bis hin zum Menschen konserviert vorliegt [Seeger *et al.*, 1998; Mundt *et al.*, 1999]. Dabei zeigte sich eine direkte Beteiligung des COP9-Komplexes an unterschiedlichen physiologischen Prozessen. So führt die Ausschaltung von CSN1 und CSN2 in *Schizosaccharomyces pombe* zu langsamerem Wachstum und erhöhter UV-Empfindlichkeit [Zhou *et al.*, 2001]. Dagegen kommt es bei *Drosophila melanogaster* zu einem Absterben im späten Larven- oder Puppenstadium, wenn die Assemblierung des Signalosoms gestört war [Freilich *et al.*, 1999]. Das Signalosom besteht aus acht Untereinheiten, deren Molekulargewicht zusammen circa 320 kDa beträgt [Schwechheimer, 2004]. Durch die Assoziation mit verschiedenen zusätzlichen Interaktionspartnern, wie zum Beispiel Kinasen, erreicht der Komplex aber bei der Aufreinigung eine Größe 450 bis 700 kDa [Uhle *et al.*, 2003].

Bisher konnten dem COP9-Komplex drei biochemische Aktivitäten zugeordnet werden: Phosphorylierung, Deneddylierung und Deubiquitinierung [Wolf *et al.*, 2003].

Durch Phosphorylierung beeinflusst der Komplex verschiedene Signaltransduktionswege [Henke *et al.*, 1999]. Hierbei werden beispielsweise c-Jun oder IκBα phosphoryliert [Seeger *et al.*, 1998]. Neben den beiden Inhibitoren des NFκB-Weges wird auch p53 durch CSN5 phosphoryliert und so für den Abbau im Proteasom markiert [Bech-Otschir *et al.*, 2002]. Die Stabilisierung von c-Jun und die Degradation von p53 werden durch eine Überexpression von CSN2 verstärkt [Huang *et al.*, 2005].

Die Deneddylierung ist wichtig für die Rolle des COP9-Komplexes als Rekrutierungsplattform für Cullin-Ubiquitinligasen, die Proteine für den Abbau im Proteasom markieren [Peng *et al.*, 2003]. Zur Aktivierung dieses Prozesses muss das Ubiquitin-ähnlichen Proteins NEDD8 an die Cullin-Untereinheit des Komplexes gebunden werden. Doch für die vollständige und dauerhafte Funktionsfähigkeit der Cullin-Ubiquitinligasen ist ein Zyklus aus Anlagerung von NEDD8 und anschließender Deneddylierung notwendig. Die Abspaltung des positiven Regulators NEDD8 wird durch den COP9-Komplex katalysiert [Wolf *et al.*, 2003; Richardson & Zundel, 2005].

Die Deubiquitinierung wird anscheinend vom Enzym USP15/Ubp12 vermittelt, das als Interaktionspartner des Signalosoms identifiziert wurde [Hetfeld *et al.*, 2005]. Dieses Protein wird auch mit der Deneddylierungs-Regulation des COP9-Signalosoms in Verbindung gebracht. Die Abspaltung des Ubiquitins kann entweder den Abbau im Proteasom verhindern oder zu einer Änderung der Aktivität von Proteinen führen, wie beispielsweise bei der Beeinflussung der DNA-Schadensreparatur durch den COP9-Komplex [Groisman *et al.*, 2003; Bondar *et al.*, 2006; Chamovitz, 2009].

Die Assemblierung des COP9-Komplexes aus seinen Untereinheiten erfordert die Interaktion der MPN-Domänen und der PCI-Domänen. Diese 150 bis 200 Aminosäuren langen Domänen wurden darüber hinaus bei der 19S Untereinheit des Proteasoms und dem Initiationsfaktor 3 beschrieben [Bech-Otschir *et al.*, 2002]. Auch in weiteren strukturellen Aspekten ähneln die Untereinheiten des Signalosoms dem Deckel des Proteasoms [Henke *et al.*, 1999; Sharon *et al.*, 2006; Sharon *et al.*, 2009]. Des Weiteren konnten in *Drosophila* bereits Wechselwirkungen zwischen CSN2 und der Proteasomenuntereinheit Rpn6 nachgewiesen werden [Lier & Paululat, 2002]. Aus diesem Grund wurde eine funktionelle Interaktion zwischen den beiden Komplexen, mit dem COP9-Komplex als alternativer Deckel des Proteasoms, postuliert [Li & Deng, 2003]. Über das Vorhandensein von Superkomplexen, die aus COP9, dem 26S Proteasom und den Ubiquitinligasen bestehen und den Abbau von Proteinen in der Zelle bestimmen, wird ebenfalls spekuliert [Huang *et al.*, 2005].

Die Strukturen des COP9-Komplexes und des Deckel-Subkomplexes des 19S Proteasoms wurden mittels 2-D-Elektronen-Mikroskopie bestimmt und miteinander verglichen. Dabei wurde festgestellt, dass beide Komplexe assymetrisch aufgebaut sind [Kapelari *et al.*, 2000; Wolf *et al.*, 2003]. Des Weiteren bestehen beide Komplexe aus zwei stabilen Modulen [Sharon *et al.*, 2009]. Darüber hinaus scheinen aber keine größeren strukturellen Ähnlichkeiten zu bestehen (Abb. 1).

COP9-Signalosom 19S Proteasom Basis-Komplex

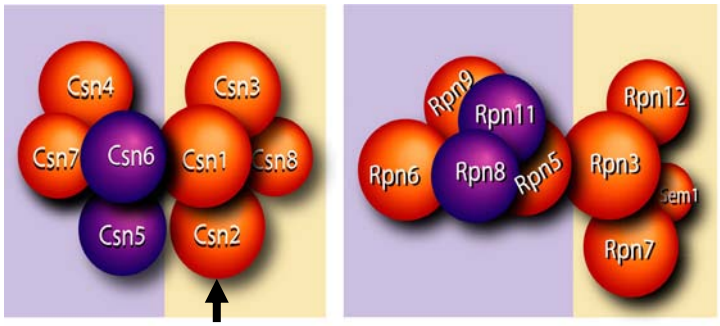

Abbildung 1. Vergleich des COP9-Signalosoms mit dem 19S Proteasom Basis-Komplex
Die Struktur der beiden Komplexe wurde mit Hilfe massenspektrometrischer Methoden aufgeklärt. Beide Komplexe bestehen aus zwei Modulen (gelb und lila hinterlegt). Trotz der hohen Sequenzhomologie zwischen den Untereinheiten bestehen größere strukturelle Unterschiede zwischen den beiden Komplexen. Die Lage von CSN2 im COP9-Komplex wird durch einen Pfeil verdeutlicht. (Abbildung modifiziert nach [Sharon *et al.*, 2009])

1.3 Das Proteasom

Neben der Degradation von Proteinen in den Lysosomen erfolgt der Abbau von Proteinen in der Zelle hauptsächlich über das Proteasom [Ferrell *et al.*, 2000]. Besonders kurzlebige Proteine, die den Zellzyklus steuern, die Transkription beeinflussen, den Zelltod auslösen oder aus einem anderen Grund in ihrer Lebensdauer strikt reguliert werden müssen, werden von diesem System abgebaut [Hershko & Ciechanover, 1998]. Zur Markierung der entsprechenden Proteine wird an diese Ubiquitin, ein 76 Aminosäuren langes Protein, katalytisch gebunden [Hershko & Ciechanover, 1998]. Die Substratspezifität wird hierbei durch eine Reihe von verschiedenen Proteinen, wie zum Beispiel Mitgliedern der Cullin-Familie, vermittelt.

Charakteristisch für das Proteasom ist die zylindrische Gestalt der rund 700 kDa schweren 20S Untereinheit, die mehrere Endopeptidase-Aktivitäten in ihrem Lumen aufweist [Driscoll, 1994]. Dieser Teil des Proteasoms kann die Proteine nicht alleine abbauen [Glickman *et al.*, 1998]. Dafür ist die Aktivierung durch das 19S regulatorische Partikel notwendig. Das 19S Proteasom weist eine reverse Chaperon-Aktivität auf, die eine Entfaltung und Translokation der Proteine in den proteolytisch aktiven Teil des Komplexes ermöglicht [Corn *et al.*, 2003]. Das regulatorische Partikel kann in zwei Subkomplexe - eine Basis und einen Deckel - getrennt werden. Dabei enthält die Basis sechs ATPasen (TRIP1, TBP1, TBP7, S4, MSS1 und p42), die die Energie für die Zerstörung der tertiären Struktur der Proteine bereitstellen, während der Deckel die Bindung der abzubauenden Proteine vermittelt [Su *et al.*, 2000]. Die 19S und 20S Subkomplexe bilden zusammen das rund 2000 kDa schwere 26S Proteasom, das für die vollständige Aktivität benötigt wird (Abb. 2) [Driscoll & Goldberg, 1990].

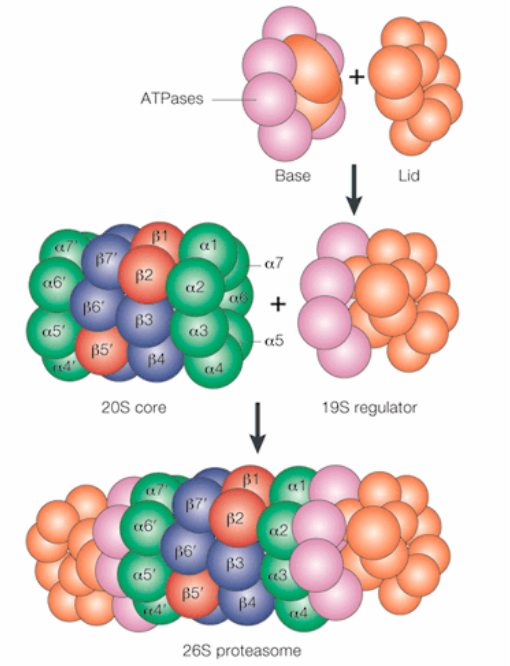

Abbildung 2. Aufbau des 26S Proteasoms und seiner Subkomplexe
Das Proteasom besteht aus dem proteolytisch aktiven 20S Proteasom und dem regulatorischen 19S Proteasom. Der 19S Subkomplex wiederum spaltet sich in die Basis, die mit Hilfe von ATPasen Proteine in das Lumen des 20S Proteasoms leitet, und in den Deckel, der für die Substratbindung und die Abspaltung der Ubiquitinreste verantwortlich ist (Abbildung aus [Kloetzel, 2001]).

1.4 Anaphase promoting complex / Cyclosome (APC/C)

Während des Zellzykluses markieren hauptsächlich zwei E3 Ubiquitinligasen, der Anaphase promoting complex / Cyclosome und der SCF-Komplex (Skp1/Cullin/F-box), Cycline mit Ubiquitin für den Abbau im 26S Proteasom. Durch diese Degradation der Cycline wird gewährleistet, dass der Zellzyklus unidirektional abläuft [Weissman, 2001; Wäsch & Engelbert, 2005]. Bisher wurden viele verschiedene Targets des APC/C beschrieben, beispielsweise Cycline (Cyclin A, Cyclin B), mitotische Kinasen (Polo-like Kinase, Aurora Kinase) und DNA-Replikationsfaktoren (Cdc6, Geminin) [Thornton & Toczyski, 2003; Lindon & Pines, 2004]. Die Substratspezifität des APC/C wird durch zwei Adaptoren vermittelt: CDH1 und CDC20. Diese Proteine binden abhängig von der Zellzyklusphase an den Komplex und unterliegen einer strikten Regulation durch posttranslationale Modifikationen und Degradation [Machida & Dutta, 2007]. Durch den APC/C wird gewährleistet, dass am Ende der Mitose alle Cycline abgebaut werden und somit die Zellen entweder in die G0-Phase übergehen oder einen neuen Zellzyklus kontrolliert beginnen können.

Der APC/C besitzt einen sehr komplexen Aufbau, der noch nicht vollständig aufgeklärt ist [Vodermaier *et al.*, 2003; Thornton & Toczyski, 2006; Herzog *et al.*, 2009]. Bisher konnten drei Teilkomplexe identifiziert werden. Der katalytisch aktive Teil besteht hauptsächlich aus dem cullin-artigen APC2 und APC11, das E2 Ubiquitinligasen binden kann [Tang *et al.*, 2001]. APC3, APC6, APC7 und APC8 bilden den zweiten Teilkomplex, der die Adaptoren CDH1 und CDC20 an den Komplex rekrutiert [Vodermaier *et al.*, 2003]. Dagegen fungieren APC1, APC4 und APC5 als Gerüst, das den katalytischen mit dem regulatorischen Teil verbindet (Abb. 3). Dieser komplexe Aufbau erschwert die molekularbiologische Analyse des APC/C-Komplex [Thornton *et al.*, 2006].

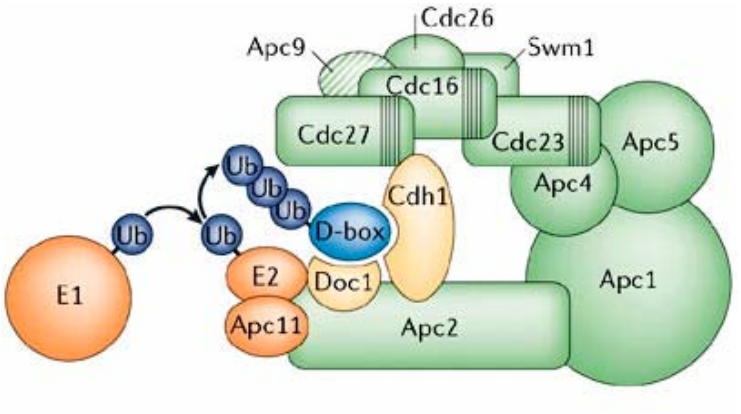

Abbildung 3: Struktur des APC/C
Die Abbildung veranschaulicht die Struktur des APC/C. Die Untereinheiten, die direkt an der Ubiquitinilierungsreaktion beteiligt sind, wurden orange gekennzeichnet. Die gelb dargestellten Proteine sind für die Substraterkennung notwendig. Dabei wird hier exemplarisch das Adaptorprotein CDH1 gezeigt. Alternativ kann auch Cdc20 an diese Stelle zwischen APC2 und CDC27 binden und damit die Substratspezifität ändern. Zusammen mit APC2 stellen die gelb und orange gekennzeichneten Untereinheiten den minimalen, katalytisch aktiven Komplex dar. APC1, APC4 und APC5 bilden das Gerüst, das diesen Subkomplex mit den TPR-Domänen-Proteinen (Tetratrico peptide repeat domain) wie Cdcd27 und Cdc16 verbindet. Es wird vermutet, dass diese TPR-Domänen-Proteinen für die Substratspezifität notwendig sind. (Abbildung aus [Peters, 2006]).

1.5 Ziele der Arbeit

Alien wurde in den letzten Jahren als Regulator verschiedener biochemischer Prozesse in der Zelle charakterisiert. Diese reichen von der Transkription über posttranslationale Modifikationen bis hin zur proteasomalen Degradation.

Um das Verständnis der biologischen Rolle von Alien in der Zelle zu erweitern, sollten in dieser Arbeit als erstes neue Interaktionspartner identifiziert werden. Dazu wurde ein Assay verwendet, der eine Kombination aus Immunpräzipitation, Erzeugung eines Peptid-Massen-Fingerabdruckes und Massenspektrometrie zur Identifizierung der gebundenen Proteine darstellt. Falsch positive und falsch negative Ergebnisse wurden durch die Untersuchung von endogen exprimierten Proteinen vermindert. Mit diesem Ansatz sollte insbesondere untersucht werden, inwiefern Alienα an Transkriptionsfaktoren aus verschiedenen Signalwegen bindet.

Des Weiteren war es ein Ziel dieser Arbeit, die hier erstmalig beschriebene Interaktion von CSN2 mit dem APC/C näher zu charakterisieren. Dabei sollte im Speziellen überprüft werden, inwiefern das COP9-Signalosom an dieser Interaktion und deren Regulation beteiligt ist. Abschließend sollte der funktionelle Einfluss von Alienα auf den APC/C untersucht werden.

2. Publikationen

2.1 Detection and identification of transcription factors as interaction partners of alien in vivo.

Kob R, Baniahmad A, Escher N, von Eggeling F, Melle C.
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In der ersten Publikation wurde ein Assay, der immunologische Ansätze und SELDI-Massenspektrometrie kombiniert, zur Identifizierung neuer Protein-Protein-Interaktionen von Alienα etabliert. Dabei wurden sowohl generelle Transkriptionsfaktoren des TFIIH Komplexes (p44 und ERCC3) als auch Coaktivatoren nukleärer Kernhormonrezeptoren (CRSP3, VDRIP und TRIP11) identifiziert. Die neu beschriebenen Bindungspartner deuten auf eine Beteiligung von Alienα sowohl bei der Transkription als auch bei der DNA Reparatur und der Zellzyklusregulation hin.
Die experimentellen Laborarbeiten wurden bei dieser Publikation ausschließlich von mir durchgeführt.

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Letter to the Editor

Detection and Identification of Transcription Factors as Interaction Partners of Alien In Vivo

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corepressor Alien, general transcription machinery, protein-protein interactions, mass spectrometry, proteomics

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ABSTRACT

Interacting proteins are often involved in the same cellular processes, and thus the identification of interacting partners of a given protein with unknown function may give insight into the physiological role of this protein. For the detection of protein-protein interactions of the corepressor Alien we used a proteomic approach comprising mass spectrometry and immunological techniques. We assessed solely endogenously expressed proteins. In this study we present for the first time that Alien is interacting within a network of proteins involved in transcriptional regulation, DNA repair, and cell cycle in vivo. In this way we detected protein interactions of Alien involving nucleophosmin, ERCC3, TRIP11, as well as CRSP3.

To get insight in the biological role of a specific protein it may be necessary to identify their interacting partners. Interacting proteins are temporally and spatially regulated and often involved in the same physiological processes. The clarification of the association of a molecular machine or regulatory factor may help to understand cellular mechanisms. Many different techniques for both in vitro as well as in vivo analysis have been used to define protein-protein interactions. These techniques are recognised as often generating fairly high numbers of both false positive and negative results.[1]

To avoid the high numbers of false results in this kind of analyses we established a procedure for the in vivo detection of protein interactions of solely endogenously expressed proteins comprising surface-enhanced laser desorption/ionization (SELDI) —mass spectrometry (MS) and immunological techniques.[2] Very recently, we showed that the endogenously expressed corepressor Alien interacts in vivo in a physical und functional manner with factors that are involved in the regulation of the cell cycle.[3] Alien was previously characterised as a corepressor for specific members of the nuclear hormone receptors such as the thyroid hormone and vitamin D3 receptor.[4] In line with this, we observed that Alien is capable to repress the transcriptional activity of members of the E2F transcription factor family suggesting a role for Alien as a corepressor also for cell cycle regulated genes.[3]

The aim of the present study was to detect in vivo further specific interacting partners of endogenously expressed Alien. Therefore, a specific anti-Alien antibody (rabbit polyclonal Pep-Ak2)[5] was coupled to protein A loaded Interaction Discovery Mapping (IDM) beads (Ciphergen Biosystems Ltd., Fremont, CA) and incubated with crude U-2OS cell extract. Previously, the immunoprecipitated material was subjected to tryptic digestion and MS as control revealing that Alien was immunoprecipitated.[3] The Alien coimmunoprecipitated captured protein complexes were eluted from the beads and assessed regarding protein composition by both SELDI-MS and SDS-PAGE. Protein signals from the immunocapturing assay using the specific Alien antibody were compared to the assay using a nonspecific antibody. Specific protein signals detected in the MS-spectra and in the SDS-PAGE were identified by peptide fingerprint mapping using SELDI MS (Fig. 1A, left panel). Hereby, among other specific signals a peak possessing a *m/z* of 36111 was detected, which corresponds very well to the MW of the alpha isoform of Alien (also named TRIP15). Alien is expressed in several isoforms one is the CSN2 subunit of the signalosome, which can be a lid of the 26S-proteasome.[6] Besides this specific Alien signal, we detected a specific signal of approximately 33 kDa in at least three assays. Additionally and as an internal control for the detecting protein interactions in vivo, we captured a signal of nearly 49.3 kDa, which corresponds very well to E2F-3. A protein interaction between Alien and E2F-3 was recently described by our group.[3] The above mentioned signals were absent in a negative control using an unspecific antibody.

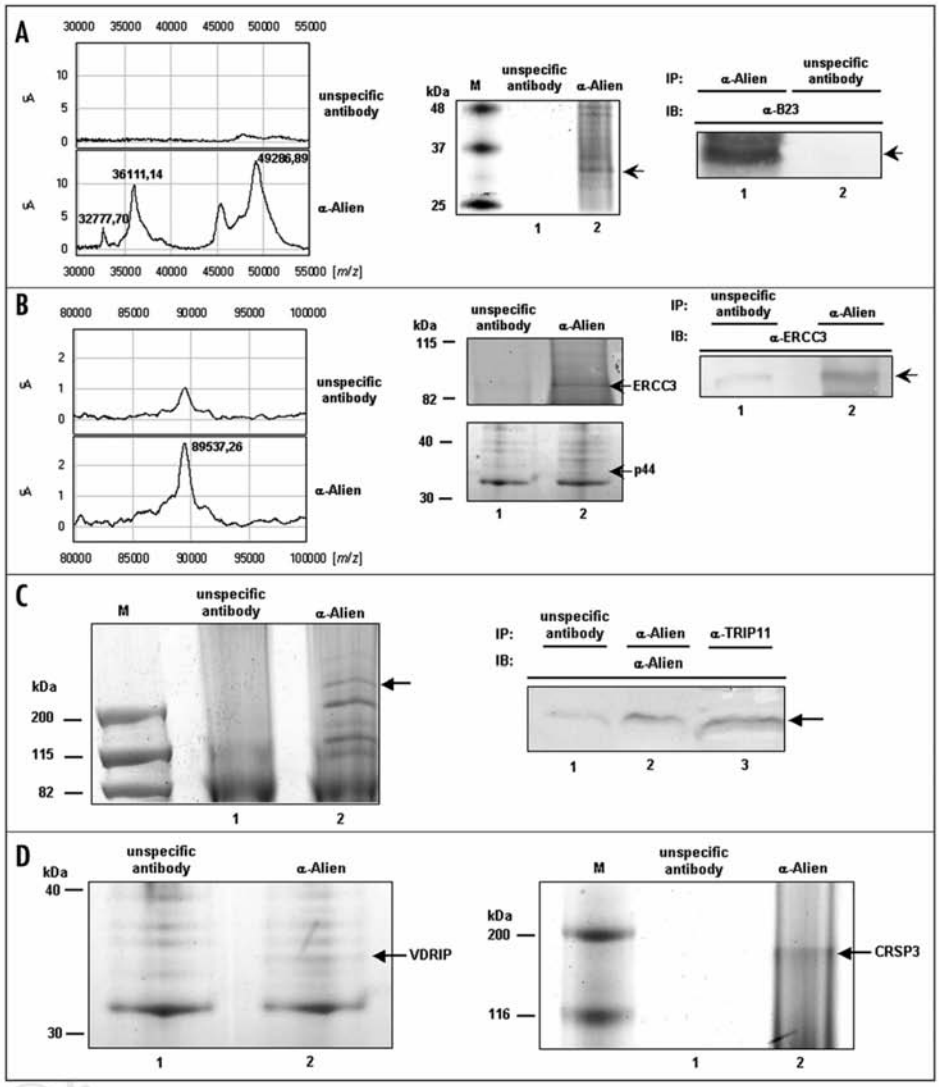

Figure 1. Detection and identification of interacting protein partners of the corepressor Alien in vivo. (A) Identification of B23 as a specific protein interaction partner of Alien. left panel: A specific anti-Alien antibody was coupled on IDM beads and incubated with U-2OS cell extract. Bound protein complexes were eluted and protein composition was analysed by SELDI-MS. A number of specific signals were detectable in the assay using the Alien antibody compared to an experiment using an unspecific antibody. Among these specific signals a signal possessing approx. 36 kDa and a signal showing approx. 49 kDa appeared which corresponds well to the molecular mass of Alien or E2F-3, respectively. Very recently, we showed a physical interaction between Alien and E2F-3.[3] Additionally, a specific signal of approx. 32.8 kDa was only detectable in the approach using the anti-Alien antibody. middle panel: Eluted proteins from the IDM beads were subsequently subjected on a SDS-PAGE for separation and a specific band of approx. 32 kDa (labelled by an arrow) was excised and used for a tryptic in-gel digestion. The peptide mass fingerprints (PMF) generated by this way were analysed by SELDI-MS and used for a database search. B23 was obtained as the best candidate (Z-score: 2.26). right panel: For the confirmation of the Alien - B23 protein interaction, coimmunoprecipitation (CoIP) experiments were used. Hereby, a specific antibody against Alien was capable to precipitate (IP) B23 from U-2OS (labelled by an arrow) shown in an immunoblot (IB) (lane 1). As a control, using an unspecific antibody, no signal corresponding to B23 was detectable (lane 2).

Figure 1 cont. (B) Identification of protein interactions between endogenously expressed Alien and subunits of the TFIIH. left panel: A signal of approx. 89.5 kDa was captured by the specific Alien antibody coupled on IDM beads from U-2OS cell extract and analysed by SELDI-MS. This signal was significantly reduced in an assay using an unspecific antibody. middle panel: The captured proteins by the Alien antibody were separated by SDS-PAGE and specific protein bands at approx. 90 kDa and 33 kDa, respectively, which were not detectable in a control using an unspecific antibody, were excised and subjected to a tryptic in-gel digestion. The generated PMFs were used for database quests which revealed ERCC3 for the 90 kDa band (Z-score: 0.81) and the p44 subunit of TFIIH for the 33 kDa band (Z-score: 1.57) as the best candidates. right panel: The protein interaction between Alien and ERCC3 was confirmed by CoIP experiments. The Alien antibody precipitated (IP) ERCC3 from U-2OS cell etract as shown in an immunoblot (IB) (lane 2). Using an unspecific antibody in a CoIP as a control, no signal correcponding to ERCC3 appeared (lane 1). (C) Alien interacts physically in vivo with the activator of the thyroid hormone receptor TRIP11. left panel: Specific captured proteins from U-2OS cell lysate using the anti-Alien antibody were analysed on SDS-PAGE. A specific protein band (labelled by an arrow) running above the 200 kDa protein marker was excised and PMF were generated. This mentioned specific protein band was absent in an experiment using an unspecific antibody. PMF of the specific protein band were used for a database quest. As best candidate, TRIP11 was revealed (Z-score: 2.22). right panel: The protein interaction of endogenously expressed Alien and endogenously expressed TRIP11 was confirmed in a CoIP experiment using the anti-Alien antibody for precipitation (IP) of TRIP11 (IB) (lane 3). As a control for the proper function of the Alien antibody, this antibody was able to precipitate Alien (lane 2). In a CoIP experiment using an unspecific antibody, TRIP11 was not quantitatively precipiated (lane 1). (D) Alien interacts in vivo with coactivators of the transcription machinery. Specific captured proteins from U-2OS cell extract by the Alien antibody were analysed on SDS-PAGE. A specific protein of approx. 35 kDa (left panel) and a specific protein band of approx. 160 kDa (right panel) was identified by generation of specific PMF and subsequent quest in a database. Both specific protein bands were absent in appendant controls using an unspecific antibody. The database quests revealed VDRIP (left panel) as the best candidate (Z-score: 1.21) or CRSP3 (right panel) as the best candidate (Z-score: 0.73), respectively.

To identify the nature of the 33 kDa signal (Fig. 1A, left panel) the proteins were eluted and subjected to SDS-PAGE. In the coomassie stained gel we detected a specific band in the range of approximately 33 kDa. Thus, we confirmed the presence of a specific Alien-interacting protein. The negative control using an unspecific antibody did not show a band at that position. This specific band was excised from the gel and was subsequently subjected to an in-gel digestion by trypsin and protein identification (Fig. 1A, middle panel). As a negative control, an empty gel piece underwent the same treatment. The digest yielded solution was spotted on a ProteinChip array and the peptide mass fingerprints (PMF) were determined by SELDI-MS. Database search (Profound; http://prowl.rockefeller.edu/prowl-cgi/profound.exe) revealed B23 (also named NPM or nucleophosmin) as the best candidate with an estimated Z-score of 2.26. To confirm this protein complex containing Alien and B23/nucleophosmin coimmunoprecipitation experiments (CoIP) were performed using crude U-2OS cell extract. In line with the previous results, we were able to precipitate B23/nucleophosmin using protein A-agarose beads with a specific anti-Alien antibody (Fig. 1A, right panel). In the negative control using an unspecific antibody coupled on the beads a signal corresponding to B23/nucleophosmin was not detectable. This suggests that endogenous Alien and endogenous B23/nucleophosmin exist in a protein complex. B23/nucleophosmin regulates cell cycle progression and stress response in hematopoietic stem/progenitor cells as well as it is able to repress the expression of negative cell cycle regulators that are associated with the G_1 phase - S phase transition.[7] B23/nucleophosmin is a nucleolar phosphoprotein that is more abundant in tumor cells than in normal resting cells. B23/nucleophosmin was identified as a substrate of CDK2/cyclin E in centrosome duplication.[8] Furthermore, B23/nucleophosmin regulates the ARF/p53 tumor suppressor pathway.[9]

Analyzing higher molecular weight proteins, we detected a specific signal of *m/z* 89537 in the MS spectrum using the specific anti-Alien antibody (Fig. 1B, left panel) and identified this signal as ERCC3 (also named XPB, xeroderma pigmentosum group B protein) using the above-described procedure (Fig. 1B, middle and right panel). ERCC3/XPB possessing a helicase activity and is a subunit of the TFIIH.[10] Additionally, we found that Alien interacts with a further subunit of the TFIIH, the p44 subunit (Fig. 1B, middle panel). Hereby, the analysis of the PMF suggested the presence of a fragment corresponding to the amino terminus and the core region of p44. TFIIH is one of the general transcription factors required for accurate transcription of protein-coding genes by RNA polymerase II. TFIIH has helicase and kinase activities, plays a role in promoter opening and promoter escape, and is also implicated in efficient activator-dependent transcription.

Using the here presented protein interaction assay also another interacting partner of Alien was detected possessing a high molecular mass, which was identified as TRIP11 (also named TRIP230) (Fig. 1C). It is not surprising that we detected this coactivator of the thyroid hormone receptor, which is described to be negatively regulated by pRB.[11] We showed recently, that Alien physically interacts also with pRB.[3] Further studies should assess whether all three proteins occur together in a protein complex and which of both Alien or pRB is responsible for the negative regulation of the thyroid hormone receptor via a direct (Alien) and/or indirect (pRB) repression.

Interestingly, a further hormone receptor binding protein, VDRIP (vitamin D receptor interacting protein; also named MED4 or DRIP36), was identified here that physically interacts with Alien (Fig. 1D). In addition, CRSP3 (also named DRIP130), a cofactor for Sp1 transcriptional activation, which is also involved in the vitamin D receptor interacting protein complex accomplishes a specific protein interaction with Alien (Fig. 1D). This suggests that similar to TFIIH interacting partners, Alien contacts two subunits of the same VDRIP complex. This also indicates that Alien does not only directly associates with the nuclear hormone receptor TR or VDR but also with protein complexes that associate with these nuclear receptors.

In the present study we show that an endogenously expressed repressor of the thyroid hormone receptor, Alien in vivo interacts physically with a number of endogenously expressed proteins, which are thought to be involved in the transcription machinery as coactivators or as general transcription factors. The current model suggests that the general transcription machinery will be recruit by the activators.[12-14] Specific components of this model including p44 and ERCC3, which are subunits of TFIIH as a member of the general transcription factor (GTF) and VDRIP and CRSP3 as coactivators are interacting partners in vivo of Alien. TFIIH is, besides its role in overall gene expression and DNA repair, also involved in transcriptional regulation by thyroid hormone receptor.[15,16] TFIIH subunit directly interacts with thyroid hormone receptor and enhances receptor-mediated transcription. It is conceivable that Alien might be function as a modulatory and procrastinatory factor for protein complexes associated with DNA-bound factors. Thus taking together, we revealed here that Alien is interacting within a network of proteins or protein complexes involved in transcriptional regulation, DNA repair, and cell cycle.

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2.2 The tumor suppressors p33ING1 and p33ING2 interact with alien in vivo and enhance alien-mediated gene silencing.

Fegers I, Kob R, Eckey M, Schmidt O, Goeman F, Papaioannou M, Escher N, von Eggeling F, Melle C, Baniahmad A.
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Diese Studie zeigt die funktionelle Interaktion von endogen expremierten Alienα mit den Tumorsuppressor-Proteinen p33ING1 und p33ING2. Die Bindung wurde *in vitro* bestätigt und eine Interaktionsdomäne konnte bestimmt werden. Hierbei wurde nachgewiesen, dass p33ING1 und p33ING2 die transkriptionelle Repression durch Alienα verstärken.
Bei dieser Publikation habe ich mit Hilfe der Immunpräzipitation, der massenspektrometrischen Analyse und des tryptischen Verdaus p33ING1 und p33ING2 als neue Interaktionspartner von Alienα *in vivo* identifiziert. Diese Ergebnisse wurden mit Co-Immunpräzipitationen der endogen exprimierten Proteine von mir bestätigt. Alle weiteren Arbeiten wurden von den anderen Autoren der Studie durchgeführt.

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The Tumor Suppressors p33ING1 and p33ING2 Interact with Alien *in Vivo* and Enhance Alien-Mediated Gene Silencing

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The tumor suppressor p33ING1 is involved in DNA repair and cell cycle regulation. Furthermore, p33ING1 is a transcriptional silencer that recognizes the histone mark for trimethylated lysine 4 at histone H3. Interestingly, expression of p33ING1 and p33ING2 is able to induce premature senescence in primary human fibroblasts. The corepressor Alien is involved in gene silencing mediated by selected members of nuclear hormone receptors. In addition, Alien acts as a corepressor for E2F1, a member of the E2F cell cycle regulatory family. Furthermore, recent findings suggest that Alien is complexed with transcription factors participating in DNA repair and chromatin. Here, using a proteomic approach by surface-enhanced laser desorption ionization and mass spectrometry (SELDI-MS) combined with immunological techniques, we show that Alien interacts *in vivo* with the tumor suppressor p33ING1 as well as with the related tumor suppressor candidate p33ING2. The interaction of Alien with p33ING1 and p33ING2 was confirmed *in vitro* with GST-pull-down, suggesting a direct binding of Alien to these factors. The binding domain was mapped to a central region of Alien. Functionally, the expression of p33ING1 or p33ING2 enhances the Alien-mediated silencing, suggesting that the interaction plays a role in transcriptional regulation. Thus, the findings suggest that the identified interaction between Alien and the tumor suppressors p33ING1 and p33ING2 reveals a novel cellular protein network.

Keywords: Corepressor • co-repressor • cellular senescence • transcriptinal silencing • tumor supressor

Introduction

The p33ING1 and p33ING2 proteins are members of the ING tumor suppressor family with five members. In human cells, all five ING proteins have been implicated in p53 function, control of cell growth/proliferation, and cancer.[1] Analyses of ING protein complexes suggest that p33ING1 and p33ING2 are associated with the mSIN3A−SAP30−HDAC complex, indicating a functional role in transcriptional repression. In line with this, p33ING1 and p33ING2 were shown to be transcriptional silencers.[2−5] Furthermore, p33ING1 and p33ING2 were shown to bind to trimethylated histone H3K4, which is a mark for chromatin-mediated gene repression.[4−6] In contrast, the majority of ING3 is associated with the hNuA4/Tip60 HAT complex, and ING4 and ING5 are associated with the HBO−histone acetyltransferase complex.[3] The ING proteins share in their C-termini a plant homeodomain (PHD) finger, a motif common to many chromatin-regulatory proteins. Both ING1 and ING2 negatively regulate cell proliferation in response to DNA damage with the participation of the tumor suppressor p53.[1] In addition, p33ING2 is involved in nucleotide excision repair via enhancement of histone H4 acetylation and chromatin relaxation.[7] Interestingly, expression of either p33ING1 or p33ING2 induces premature senescence,[2,8] indicating a further functional similarity between p33ING1 and p33ING2. In addition, the association of p33ING1 and p33ING2 with cancer was reported through identification of mutations of both ING genes. Most of the point mutations were described in the PHD domain; also, loss of heterozygosity in sporadic basal cell carcinomas that involves the p33ING2 and SAP30 genes were reported[9] as well, as reduced expression of p33ING1 and p33ING2 in human cancer were observed.[10−12]

Alien is a highly conserved protein and was characterized as a corepressor for a variety of nuclear hormone receptors including the thyroid and vitamin D3 receptors TR and VDR.[13−15] Alien enhances nuclear hormone receptor-mediated transcriptional silencing and possesses intrinsic transcriptional

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silencing function. One mechanism of Alien-mediated silencing was shown to be HDAC-dependent. In addition, recently, we discovered Alien as a corepressor for the cell cycle regulator E2F1, inhibiting its transcriptional activity.[16] Several Alien isoforms have been reported *in vivo*,[17] the alpha isoform of Alien, also named TRIP15, and the beta form also known as CSN2, a subunit of the signalosome, which can also be a lid of the 26S-proteasome.[18] The Alien alpha represents a C-terminal deletion of the Alien beta/CSN2 isoform generated from the same genetic locus. The functional differences between the isoforms are not yet clear. Here, by performing a protein−protein complex detection assay, we could show that Alien is interacting *in vivo* with the tumor suppressor p33ING1. On the basis of this finding, we tested the notion whether p33ING2 also interacts with Alien and whether this interaction might be functionally linked to transcriptional repression.

Materials and Methods

Protein−Protein Complex Detection Assay. The protein−protein complex detection assay was described elsewhere.[16,19] Briefly, an antibody against Alien (rabbit polyclonal Pep-Ak2[13]) or normal rabbit IgG (Pepro Tech, Inc., Rocky Hill, NJ) as negative control was coupled with Interaction Discovery Mapping (IDM) beads (Ciphergen Biosystems Ltd., Fremont, Ca) and incubated with 100 μL of crude U-2OS cell extract for 1 h at 4 °C in an end-over-end mixer. Bound proteins were eluted from the IDM beads using 25 μL of 50% acetonitrile/0.5% trifluoroacetic acid. Five microliters of the eluted samples were applied to the activated, hydrophobic surface of an H50 ProteinChip Array (Ciphergen Biosystem, Inc., Fremont, CA) and analyzed in a ProteinChip Reader (series 4000; Ciphergen, Biosystems Ltd., Fremont, Ca) according to an automated data collection protocol by SELDI-MS. This includes an average of 265 laser shots to each spot with a laser intensity of 2300 nJ and 3500 nJ, respectively, dependent on the measured region (low = 2.5−20 kDa and high = 20−200 kDa, respectively) and an automatically adapted detector sensitivity.

Peptide Fingerprint Mapping. Peptide fingerprint mapping was carried out as described elsewhere.[16] In brief, the volume of eluted samples was reduced to a maximum of 10 μL using a speed-vac (ThermoServant) and subjected to SDS-PAGE for separation of proteins followed by staining with Simply Blue Safe Stain (Enhanced Coomassie, Invitrogen). Specific gel bands were excised, destained, and dried followed by rehydration and digestion with 10 μL of a trypsin solution (0.02 μg/μL; Promega) at 37 °C overnight. The supernatants of the in-gel digestions were applied directly to NP20 arrays (Ciphergen, Biosystems Ltd., Fremont, Ca). After addition of the matrix (CHCA), peptide fragment masses were analyzed using the ProteinChip Reader, series 4000 instrument. A standard protein mix (all-in-1 peptide standard mix; Ciphergen, Biosystems Ltd., Fremont, Ca), including Arg8-vasopressin (1082.2 Da), somatostatin (1637.9 Da), dynorphin (2147.5 Da), ACTH (2933.5 Da), and insulin beta-chain (3495.94 Da), was used for calibration. Proteins were identified using a search of the fragment masses in a publicly available database (http://129.85.19.192/profound_bin/WebProFound.exe).

Co-Immunoprecipitation (CoIP). The CoIP experiments were carried out as described.[16] Briefly, specific antibodies against p33ING1 or p33ING2 (Supporting Information), separately, or, as negative control, normal rabbit IgG were bound on protein A-agarose beads. p33ING-specific antibodies were obtained from immunization of rabbits by bacterially expressed

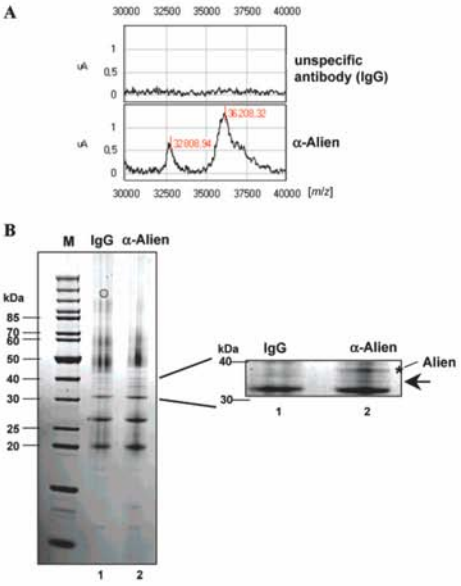

Figure 1. Detection and identification of the tumor suppressor protein p33ING1 as specific interacting protein of Alien by protein−protein complex detection assay. (A) An anti-Alien antibody was coupled with IDM beads and incubated with U-2OS cell extract. Bound proteins were analyzed by SELDI-MS. Among a signal corresponding to Alien, a signal at approximately 32.5 kDa was detectable which is absent in the assay using an unspecific antibody. (B) Eluted proteins from IDM beads were subsequently subjected on a SDS-PAGE for separation, and a specific band at approximately 33 kDa (labeled by an arrow) was excised and used for a tryptic in-gel digestion. The generated peptide mass fingerprints were analyzed by SELDI-MS and used for a database quest. As the best candidate, p33ING1 was obtained (see also Supporting Information figure). As a control, a specific band in the range of approximately 36 kDa was revealed in a database search as Alien (labeled by asterisk).

p33ING1 or p33ING2 (Eurogentec). The Alien antibody was described earlier.[13] Crude extract (100 μL) from U-2OS cells was incubated with the antibody-loaded beads for 1 h at 4 °C. Then, the resins were washed three times with CoIP buffer containing 20 mM HEPES/KOH, pH 8.0, 50 mM KCl, 0.1 mM EDTA, and 0.05% CHAPS. Bound proteins were subjected to 10% SDS−PAGE and detected by immunoblotting. Signals in immunoblots were detected indirectly by staining with NBT/BCIP using a secondary antibody linked with an alkaline phosphatase.

GST−Pull-Down Assays. The assay was described earlier.[14] pGST-linker, GST-Alien, and deletions thereof were described earlier.[13,14] Bacterial expression vectors for GST-p33ING1 and GST-p33ING2 were kindly provided by M. Serrano, Madrid, Spain. The GST−ING fusion proteins were obtained from transformed BL21 *Escherichia coli* strain and the GST−Alien fusion protein expression in *E. coli* HB101 cells. The induction of GST or GST-fusion gene expression was performed with 0.2 mM IPTG for 3 h at 37 °C. Purification was achieved with glutathione beads (Amersham Biosciences). Bound GST-

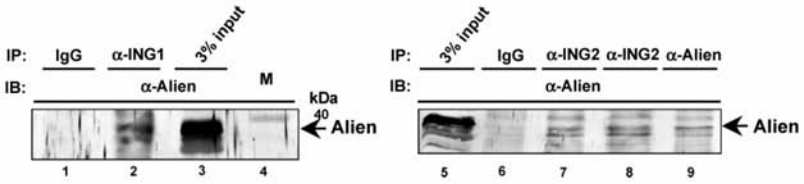

Figure 2. p33ING1 and p33ING2 as novel *in vivo* interaction partners for Alien. For confirmation of the p33ING1−Alien interaction, co-immunoprecipitation experiments were used. Thereby, a specific antibody against p33ING1 was capable to immuneprecipitate (IP) Alien from U-2OS cells (labeled by arrow) shown in an immunoblot (IB) (left panel, lane 2). Using an unspecific antibody as control, no signal corresponding to Alien was detectable (left panel, lane 1). In additional CoIP experiments, two different and specific anti-p33ING2 antibodies were able to precipitate (IP) Alien from U-2OS cells (labeled by arrow) shown in an immunoblot (IB) (right panel, lanes 7 and 8) compared to the CoIP experiments using an unspecific antibody as control (right panel, lane 6).

proteins were washed three times with NETNM-buffer (20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, and 0.5% fat free milk (Carnation, MD). *In vitro* translated, ^{35}S-methionine labeled proteins were generated using the TNT-kit (Promega) according to the manufacturer's protocol.

Cell Culture and DNA Transfection. HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C/5% CO_2 and were transfected with the indicated amount of plamids by the $CaPO_4$-method.[14] pCMV-LacZ was included as an internal control and was used for normalization.

Results

Detection of p33ING1 and p33ING2 as Novel *in Vivo* Interaction Partner for Alien. Alien possesses transcriptional repressive activity, and thus, it would be interesting to identify specific targets. For this reason, we performed a protein−protein complex detection assay to detect interacting proteins of endogenously expressed Alien in crude extracts of U-2OS cells. Alien-interacting proteins were immunoprecipitated with a specific anti-Alien antibody coupled with IDM beads followed by elution of the captured proteins and analysis of the protein complex composition using SELDI-MS (Figure 1A). Hereby, among a specific signal possessing a m/z of 36 208 that corresponds to Alien alpha, we detected a peak with a m/z of approximately 32.5 in at least three independent assays. Both signals that derived from Alien alpha and were in the range of 32.5 kDa were absent in the negative control using an unspecific antibody. For identification of the 32.5 kDa signal, we subjected the eluted proteins to SDS-PAGEs. In the coomassie-stained gel, we detected, among others, a specific band in the range of approximately 33 kDa. Thus, we confirmed the presence of a specific Alien-interacting protein. The negative control using an unspecific antibody did not show a band at that position (Figure 1B). This specific band was excised from the gel and was subsequently subjected to an in-gel digestion by trypsin and protein identification. As a control, an empty gel piece underwent the same treatment. The digest solution was spotted on a NP20 array, and the peptide mass fingerprints were determined by the ProteinChip Reader. Database searches (Profound; http://129.85.19.192/profound_bin/WebProFound.exe) revealed the tumor suppressor protein p33ING1 as the best candidate with an estimated Z-score of 2.27 (Supporting Information Figure S1). Another specific band in the range of approximately 36 kDa (Figure 1B, labeled by asterisk) was also treated as mentioned above and revealed Alien by a database search (data not shown).

To confirm this protein complex containing p33ING1 and Alien, co-immunoprecipitation experiments (CoIP) were performed using crude U-2OS cell extract. In line with the previous results, we were able to precipitate Alien using protein A-agarose beads with a specific anti-p33ING1 antibody (Figure 2, left panel). A signal corresponding to Alien was not detected in negative control experiments using a bead-bound, unspecific antibody. These data indicate that Alien is complexed with p33ING1.

On the basis of sequence and some functional similarities between p33ING1 and p33ING2, we inquired if Alien also interacts with p33ING2. To test this hypothesis, co-immunoprecipitation experiments using a specific anti-ING2 antibody were performed with crude U-2OS cell extract. This anti-ING2 antibody was able to precipitate Alien (Figure 2, right panel). This suggests that an *in vivo* complex containing Alien and ING2 also exists. Thus, Alien interacts with the tumor suppressor proteins p33ING1 and p33ING2.

Binding of p33ING1 and p33ING2 to Alien *in Vitro* Requires a Central Domain of Alien. First, we wondered whether Alien interacts *in vitro* directly with both p33ING1 and p33ING2. For that purpose, we bacterially expressed and affinity-purified GST−ING1 and GST−ING2 fusion proteins, or GST alone, as a negative control. *In vitro* translated and ^{35}S-labeled Alien was added. After stringent washes, the bound proteins were loaded on a SDS-PAGE, and the radioactive-labeled Alien was detected by fluorography (Figure 3A,B). A signal was detected specifically in the GST-p33ING1 or p33ING2 lanes, whereas no signal appeared in the GST control. Thus, these data suggest that Alien interacts with both p33ING1 and p33ING2 *in vitro*. Deletion analyses of p33ING1 suggest that the full-length p33ING1 (aa 1−279) protein is required for efficient interaction with Alien (Figure 3C−E).

In addition, we wanted to identify the interaction domain of Alien and used for that purpose the *vice versa* experimental setup using bacterially expressed and affinity purified Alien or Alien deletions as the GST-fusion and *in vitro* translated and radioactive-labeled p33ING1 or p33ING2. Both p33ING1 and p33ING2 showed a similar interaction pattern with Alien and Alien deletions (Figure 4A). Whereas Alien beta/CSN2 (aa 1−444) and the Alien alpha isoform (aa 1−305) interact with p33ING1 and p33ING2, the amino-terminal part of Alien lacked interaction. This region harbors a silencing domain of Alien.[14] Interestingly, the central region encompassing the amino acids

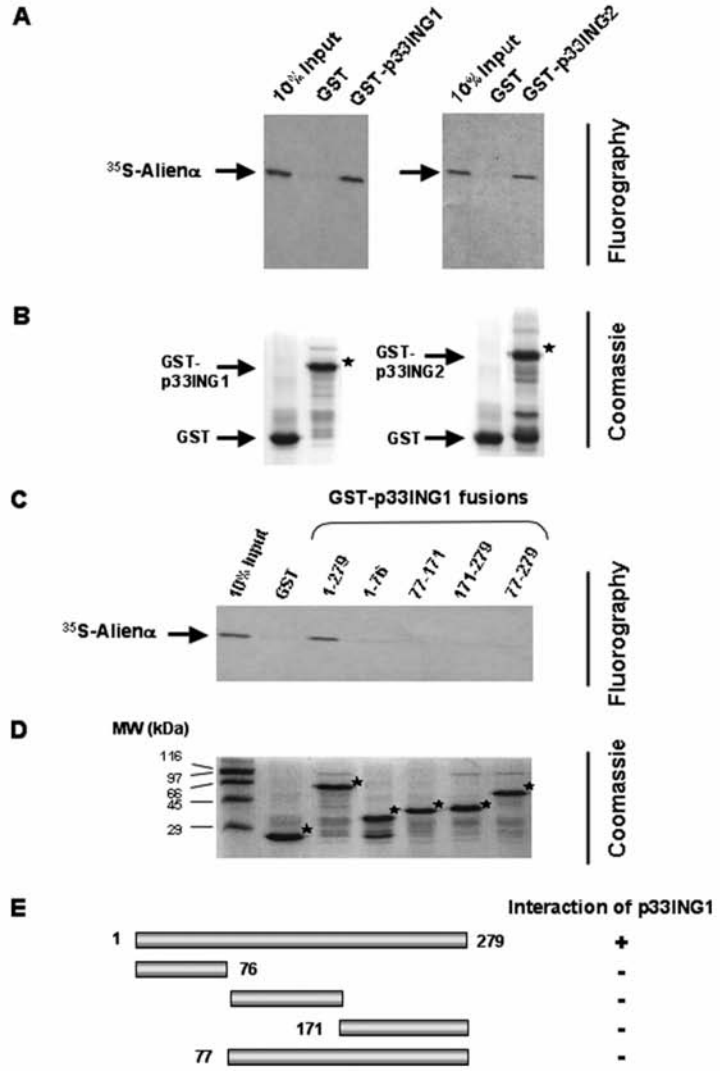

Figure 3. p33ING1 and p33ING2 interact *in vitro* with Alien GST−pull-down assays using bacterially expressed and affinity-purified p33ING1 or p33ING2 as GST fusions that were incubated with *in vitro* translated and radiolabeled Alien. (A) After stringent washes, proteins were separated on SDS-PAGE, and the radiolabeled proteins were visualized by fluorography. The input lane corresponds to 10% of the *in vitro* translated material used for GST−pull-down assays. Arrows indicate the migration of Alien. (B) Coomassie staining of the GST, GST-p33ING1, or p33ING2 to indicate the proteins used. (C) GST, GST-p33ING1, or deletions thereof were employed in the GST−pull-down assay with *in vitro* translated and radiolabeled Alien. (D) Coomassie staining of the GST, GST-p33ING1, and GST-p33ING1 deletions to indicate the amounts of proteins employed. (E) Schematic view on the p33ING1 deletions and the summarized data obtained with the GST−pull-down assay.

(aa) 128−305 of Alien was sufficient to interact with both p33ING1 and p33ING2 (Figure 4A). The intensity of the bands suggests that this region mediates the interaction of the p33ING proteins with Alien. Further deletions of Alien including the C-terminal silencing domain lacked interaction. As control for comparisons of the Alien mutants, the amount of the GST-

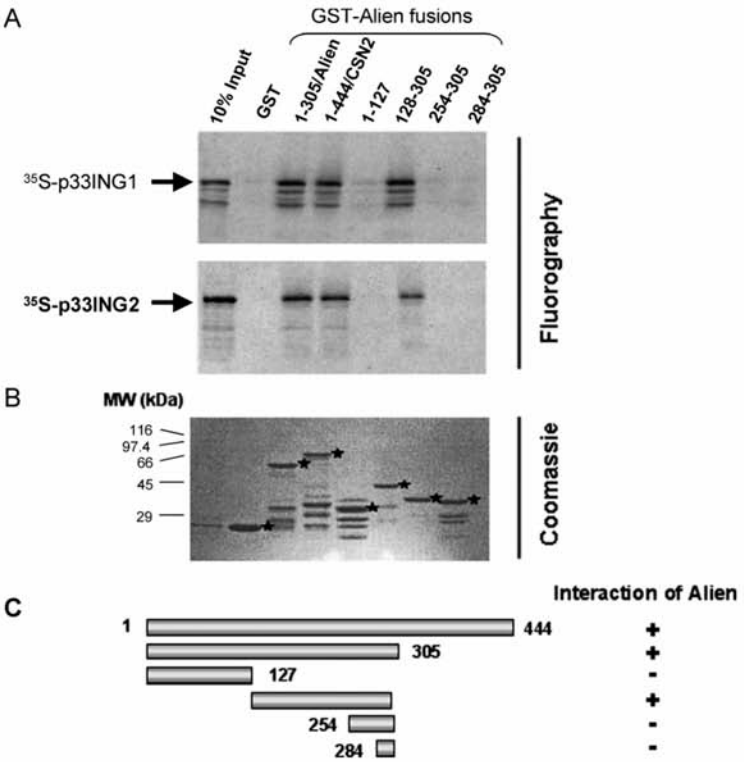

Figure 4. The central part of Alien is sufficient to interact with p33ING1 or p33ING2 GST−pull-down assays using bacterially expressed and affinity-purified GST, GST-Alien, or GST-Alien deletion mutants in a similar experimental setup as in Figure 3. The numbers indicate the amino acid endpoints of Alien and deletions thereof. Alien 1−444 is the Alien beta isoform corresponding to the CSN2 subunit of the signalosome, and Alien 1−305 is the alpha isoform.[17] Arrows indicate the migration of the p33ING proteins. (A) GST−pull-down assays with *in vitro* translated and ^{35}S-methionine labeled p33ING1 (upper panel) or p33ING2 (lower panel). (B) Coomassie staining of the SDS gel to visualize the amounts of GST-Alien fusions employed in the GST−pull-down assays. The migration of the full-length proteins are indicated by an asterisk. (C) Summary of the employed Alien mutants and the data obtained with the GST−pull-down assay.

fusion proteins were visualized by Coomassie staining (Figure 4B). The summary of the deletions analyses is shown schematically in Figure 4C. Thus, these analyses confirmed the *in vivo* findings and suggest that Alien interacts with both tumor suppressor proteins p33ING1 and p33ING2 also *in vitro* and indicate a direct interaction between Alien and p33ING1 and p33ING2.

p33ING1 and p33ING2 Mediate Gene Silencing through Alien. To reveal a possible functional role, we hypothesized that, since both factors play a role in transcriptional regulation, the interaction of p33ING1 and p33ING2 could influence the silencing function of Alien. Therefore, we used the established Gal-fusion system to test for silencing function with Gal-Alien as the DNA binder and overexpressed each p33ING1, p33ING2, or the empty mammalian expression vector (control; C). As the reporter, we used the UAS4x-tk-Luc containing four Gal4 binding sites in front of the thymidine kinase promoter. As a control, we used the expression vector for Gal alone, and the obtained values were β-galactosidase-normalized to the internal control obtained with cotransfecting pCMVLacZ (Figure 5). To test for silencing function, conditions with weak Alien-mediated silencing were chosen. Expression of either p33ING1 or p33ING2 had no significant effect when Gal alone was used. However, when the Gal-Alien fusion was used, both p33ING1 and p33ING2 enhanced the Gal-Alien-mediated silencing function compared to the control vector in a synergistic manner (Figure 5). As a further control, the Alien deletion mutant Gal-Alien 1−127 was employed that did not interact *in vitro* and did not show an effect upon coexpression either of the p33ING factors. Thus, these data suggest that both p33ING1 and p33ING2 can enhance Alien-mediated silencing.

Discussion

Because of the analysis of the composition of protein complexes, it might be possible to gain insight in cellular processes in which specific proteins are involved. The inves-

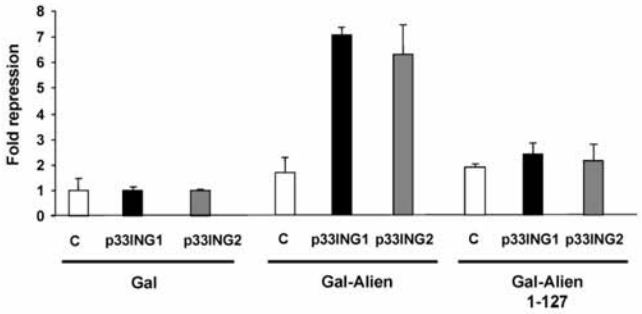

Figure 5. The expression of either p33ING1 or p33ING2 enhances Alien-mediated gene repression. HEK293 cells were cotransfected with the reporter (UAS4xtk-Luc; 1 μg), the mammalian expression vectors for Gal alone, Gal-Alien, or Gal-Alien 1−127 (2 μg) and for p33ING1, p33ING2, or the corresponding empty vector (1 μg) together with the pCMV-LacZ (0.05 μg) plasmid for normalization of transfection efficiency. After normalization of the obtained luciferase values, the data were plotted as "Fold repression" relative to the Gal-control. Error bars represent the deviation of the mean of triplicate experiments.

tigation of protein complexes of solely endogenously expressed proteins avoids the tendency to detect false-positive protein−protein interactions of examinations performed *in vitro*. We describe here a novel protein−protein interaction pathway in which we link the tumor suppressors p33ING1 and p33ING2 with the corepressor Alien. In general, corepressors are acting as transcription factors that mediate or enhance the gene silencing of DNA-bound transcription factors.[20] A common characteristic of corepressors is that they act at the chromatin level and repress transcription by interacting directly or indirectly with DNA-bound transcription factors. Alien has been shown to be a corepressor for several nuclear receptors such as TR, VDR, and DAX1 mediating gene repression. Recently, Alien was also shown to be a corepressor for the cell cycle regulator E2F1.[16,17] and we revealed also the physical interactions of Alien with a number of different factors involved in transcription and DNA repair comprising subunits of the TFIIH and CRSP3/DRIP130.[22]

One mechanism of gene repression is suggested to occur at the chromatin level and in part through the recruitment of HDAC activity at regulatory sites. Interestingly, both the p33ING proteins and Alien were previously described to interact with the mSIN3A−SAP30−HDAC complex.[3,14,21] Also, both the p33ING proteins and Alien were shown to possess transcriptional silencing function recruiting HDAC activity.[2,3,21] In addition, both p33ING and Alien were shown to be recruited to chromatin.[4,5,14] On the basis of the studies here, we could extent our knowledge of the cellular protein interaction network of Alien revealing interaction with the tumor suppressors p33ING1 and p33ING2.

Here, we reveal that both p33ING and Alien interact in cells *in vivo* and *in vitro*, and that Alien interacts directly with either p33ING1 or p33ING2. The transfection data suggest that the p33ING proteins could serve as corepressors for Alien. Whether Alien serves as corepressor for the p33ING proteins was not detectable within this assay (Supporting Information Figure 3S), which could be due to higher levels of endogenous Alien in these cells. The increase in silencing function, when both factors are coexpressed, suggests the interaction plays a role in transcriptional repression. Mechanistically, the interaction of both the p33ING and Alien might increase the frequency of recruitment or stabilize the chromatin association of the mSIN3A−SAP30−HDAC complex to chromatin and thus lead to an enhanced gene repression. Since it is generally assumed that all target genes of Alien are not necessarily and automatically also target genes for p33ING1 or p33ING2, specific target genes that are regulated by both Alien and ING need to be identified. Those common target genes could be identified by global microarray in combination with siRNA approaches.

However, it cannot be excluded that the interaction of Alien with p33ING1 and p33ING2 might play a role within other cellular contexts or pathways. Since both p33ING1 and p33ING2 were implicated to play a role in UV-damage repair and cellular senescence, we do not exclude that the interaction between Alien and the p33ING proteins might play also a role in these cellular functions. In line with this, Alien was also found as *in vivo* interacting partner with factors that are involved in DNA repair such as XPB, xeroderma pigmentosum group B protein, and the p44 subunit of TFIIH.[22] This linkage has the notion that, in addition to gene repression, the interaction of Alien with the p33ING proteins might play a role in DNA repair. Also, it can be hypothesized that the function of the p33ING proteins might be regulated by the signalosome. Since one function of the signalosome is the regulation of protein levels, the interaction of the p33ING proteins with Alien might lead to control of p33ING protein levels.

Together with the findings shown here, it suggests that Alien is not only a corepressor restricted to nuclear hormone receptors but indicates a much broader cellular interaction network.

Abbreviations: aa, amino acids; CoIP, co-immunoprecipitation; GST, glutathione *S*-transferase; HDAC, histone deacetylase; IB, immunoblot; ING1, ING2, inhibitor of growth 1, 2; IP, immunoprecipitation; MS, mass spectrometry; SELDI, surface-enhanced laser desorption ionization; TR, thyroid hormone receptor; VDR, vitamin D3 receptor.

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Supporting Information Available: Figures showing: Figure 1S, the trypsin digest solution spotted on a NP20 array and the peptide mass fingerprints determined by the ProteinChip Reader; Figure 2S, Western blot analysis of trans-

fected HEK293 cells with either the empty control vector (C) or expression vectors for p33ING1 or p33ING2; Figure 3S, the repression mediated by ING factors is not significantly enhanced by coexpressing Alien. This material is available free of charge via the Internet at http://pubs.acs.org.

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2.3 Regulation of the anaphase-promoting complex by the COP9-signalosome.

Kob R, Kelm J, Posorski N, Baniahmad A, von Eggeling F, Melle C.
Cell Cycle. 2009 Jul 1;8(13):2041-9.

In einer weiteren Protein-Protein-Interaktionstudie wurde eine Wechselwirkung des CSN2 mit dem Anaphase promoting complex / Cyclosome nachgewiesen. Damit konnte ein neuer Regulationsmechanismus des proteasomalen Abbaus durch CSN2 gezeigt werden. Durch diese Beeinflussung kommt es zu einer veränderten Degradation verschiedener Zellzyklusregulatoren, wodurch eine erhöhte genetische Instabilität erzeugt wird.
Die experimentellen Laborarbeiten wurden weitestgehend von mir durchgeführt. Ausgenommen davon sind die Experimente, die auf einem knock-down von CSN5 beruhen und von der von mir betreuten Diplomandin Juliane Kelm bearbeitet wurden. Die genetische Instabilität wurde von Nicole Posorski mit Hilfe des 50k Mikroarrays von Affymetrix gemessen. Die verwendeten Plasmide wurden von Prof. Aria Baniahmad zur Verfügung gestellt.

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Report

Regulation of the anaphase-promoting complex by the COP9 signalosome

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Key words: 26S proteasome, COP9 signalosome, CSN2, anaphase-promoting complex/cyclosome, Alien, protein-protein interactions

The COP9 complex (signalosome) is a known regulator of the proteasome/ubiquitin pathway. Furthermore it regulates the activity of the cullin-RING ligase (CRL) families of ubiquitin E3-complexes. Besides the CRL family, the anaphase-promoting complex (APC/C) is a major regulator of the cell cycle. To investigate a possible connection between both complexes we assessed interacting partners of COP9 using an in vivo protein-protein interaction assay. Hereby, we were able to show for the first time that CSN2, a subunit of the COP9 signalosome, interacts physically with APC/C. Furthermore, we detected a functional influence of the COP9 complex regarding the stability of several targets of the APC/C. Consistent with these data we showed a genetic instability of cells overexpressing CSN2.

Introduction

The temporal regulation of degradation of damaged or misfolded as well as short-lived proteins in a specific manner is an essential feature of eukaryotic growth and development. Hereby, the SCF (Skp1/Cullin/F-box) complex, an E3 ubiquitin ligase, marks specific proteins with ubiquitin (Ub) for destruction by the 26S proteasome.[1] As a protein complex, that possesses structural similarities to a subunit of the 26S proteasome, the COP9 signalosome was discovered.[2] The COP9 signalosome (also named CSN) was first identified in Arabidopsis and comprises eight conserved subunits, CSN1-CSN8, in all eukaryotic cells.[3] These subunits bear remarkable homologies to the 19S lid of the 26S proteasome as well as to the translational initiation complex eIF3 and are postulated recently to possess an as yet undetermined function in protein degradation.[4,5] Further, there are suggestions that the COP9 complex is able to substitute the 19S lid functionally.[6] The COP9 complex is able to interact with the 19S regulatory complex, replace or interact with the 19S lid resulting in a supercomplex containing 26S proteasome and COP9 signalsosome and E3 ligases.[7] There are data available indicating that the CSN subunits interact directly with the 26S proteasome. In Arabidopsis as well as in Drosophila, CSN1 interacts with Rpn6, a subunit of the 19S lid.[8,9] Furthermore, the COP9 signalosome competes with the 19S lid for binding to the 26S proteasome in vitro.[10] Additionally, it has been shown that subunits of COP9 interact with different subunits of cullin dependent E3 ligases. Hereby, the CSN5 subunit which possesses a metalloproteinase activity is able to remove Nedd8 from cullins.[11] Cycles of neddylation and deneddylation of cullins seem to regulate the ubiquitinating activity of the cullin-based Ub ligases.[12] As yet, there are no data available showing an interaction of the COP9 signalosome with the cullin like protein APC2. Besides the SCF complex, the anaphase promoting complex (APC/C) is the second major E3 ligase mediating degradation of cyclins during cell cycle.[13] Since APC/C especially regulates the mitosis and the segregation of centrosomes it has a great impact on the genomic stability.[14-16] Recently, it was revealed that COP9 is essential for cell cycle progression and genomic stability in Arabidopsis.[17]

In a former study, we identified interacting proteins of the co-repressor Alien, an isoform of the CSN2 subunit of COP9 signalosome, under in vivo conditions.[18] The aim of the present study was to detect and analyze functional interactions between the COP9 complex and proteins involved in cell cycle as well as maintenance of genomic stability to get a detailed insight in cell cycle regulation and cell proliferation.

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Results

CSN2 interacts with the anaphase-promoting complex in vivo. CSN2 as a subunit of the COP9 signalosome was shown to interact with several factors of the ubiquitin-proteasome pathway.[7] Recently, it was shown that the signalosome binds to cullin 1–4 and affects their function.[19-23] To extend our knowledge about this network, it is necessary to detect further interaction partners of COP9. For this reason, we performed a protein-protein interaction assay. Therefore, we precipitated an endogenously expressed subunit of COP9, CSN2, from crude U2OS cell extract using a specific antibody against CSN2. In order to recognize unspecific precipitated proteins we used as a negative control a rabbit

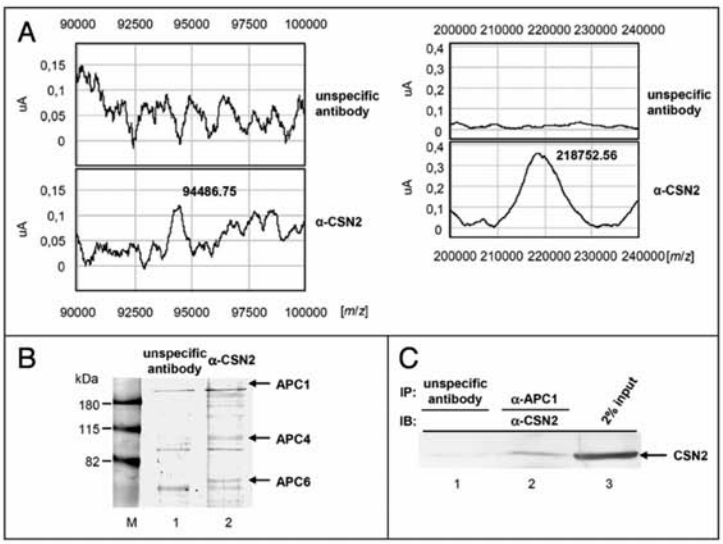

Figure 1. Endogenous CSN2 interacts with several subunits of the APC/C in vivo. (A) In protein-protein complex detection assays, a specific anti-CSN2 antibody was coupled on IDM beads and incubated with crude U2OS cell extract. Bound protein complexes were eluted and analyzed by SELDI-MS. A number of specific signals were detectable in the assay using the specific CSN2 antibody compared to an unspecific antibody. Among other specific signals two signals possessing 218.7 kDa and 94.5 kDa, respectively, were detected which correspond well to the molecular mass of APC1 and APC4, respectively. (B) The eluate was separated using an SDS-PAGE. Three specific protein bands (labeled with arrows) were excised from the coomassie stained gel and subjected to tryptic in-gel digestion. The generated peptide mass fingerprints (PMF) were analyzed by SELDI-MS and compared to an online database. Hereby, APC1 (at approx. 220 kDa), APC4 (at ~100 kDa) and APC6 (at ~70 kDa) were obtained as the best candidates. (C) The CSN2–APC1 protein interaction was confirmed by reverse CoIP experiments. CSN2 (labeled with an arrow) was co-immunoprecipitated (IP) from crude U2OS cell extract by a specific APC1 antibody as detected by an immunoblot (IB) (lane 2) compared to a negative control using an unspecific antibody (lane 1). A faint band in the negative control (lane 1) was unspecific precipitated by the non-specific antibody. Using a densitometrical analysis, this unspecific band represents only 19% of the protein band corresponding to CSN2 (lane 2) which was specific co-precipitated by the anti-APC1 antibody.

pre-immune serum. Captured proteins were eluted and analyzed using SELDI-MS (Fig. 1A). A specific precipitated signal possessed an *m/z* of 218,752 which roughly corresponds to the molecular weight of the anaphase-promoting complex subunit APC1. This signal was absent in the negative control. To identify the 219 kDa signal we subjected the eluted proteins to SDS-PAGE and detected a specific band in the range of ~220 kDa. Beside this specific band we detected further specific bands at ~100 kDa and at ~70 kDa (Fig. 1B). The negative control using a rabbit pre-immune serum did not reveal bands at these positions. Thus, we confirmed the presence of specific CSN2-interacting proteins. These specific bands were excised from the gel and subsequently subjected to an in-gel digestion by trypsin and protein identification. As control, an empty gel piece underwent the same treatment. The digest yielded solution was spotted on a Au array and the peptide mass fingerprints (PMF) were determined by SELDI-MS. Database searches (Profound; prowl.rockefeller.edu/prowl-cgi/profound.exe) revealed APC1 (Z-score: 2.39; supplemental Fig. 1; theoretical MW: 216,500 Da), APC4 (Z-score: 0.76; supplemental Fig. 2; theoretical MW: 92,116 Da), and APC6, also named CDC16 homolog (Z-score: 0.73; supplemental Fig. 3; theoretical MW: 71,656 Da) as the best candidates for the three detected interaction partners. APC1, APC4 as well as APC6 are subunits of the anaphase-promoting complex (APC/C). The APC/C is involved in protein degradation of cell cycle regulating factors. To confirm the presence of a protein complex containing CSN2 and APC1 we performed a co-immunoprecipitation experiment (CoIP). In line with the previous results, we were able to co-precipitate CSN2 using a specific APC1 antibody from U2OS cells (Fig. 1C). Additionally, we were able to coprecipitate APC1 by a specific antibody against CSN2 in a reciprocal CoIP (Fig. 2, lanes 1 and 2). The CSN2/APC1 complex appeared in a cell cycle dependent manner (Fig. 2). These data suggest that endogenous CSN2 exists together with endogenous APC/C, at least transiently, in one and the same stable protein complex.

CSN2 binding to APC/C but not to the base of the 19S proteasome is independent by interaction to COP9. The base, a sub-complex of the 19S proteasome, seems to interact with different subunits of the COP9 signalosome.[9,10] In order to uncover the biological function of the interaction of COP9 with

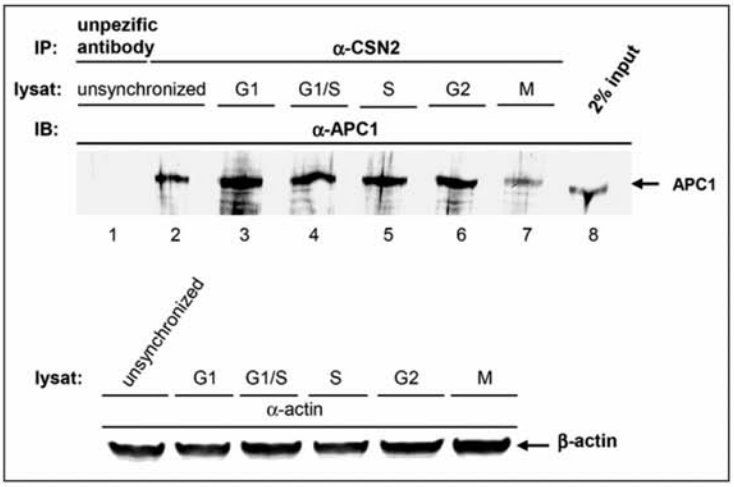

Figure 2. Interaction between CSN2 and APC1 is cell cycle dependent. U2OS cells were synchronized at different cell cycle stages. Synchronization in specific phases was achieved by following treatments. G_1 phase: 0.5 mM mimosine 24 h. G_1/S phase: 2 mM hydroxy urea over night. S phase/G_2 phase: U2OS cells were blocked in G_1 by serum starvation for 24 h following addition of fresh medium containing FBS supplemented with 2.5 mM thymidine over night. M phase: cells were blocked in mitosis by incubation with 100 ng/ml nocodazol for 16–18 h. Cells were lysed using lysis buffer and cleared by centrifugation (15 min; 15,000 rpm) at 4°C. The supernatants corresponding to protein extracts of different cell cycle stages were immediately used for CoIP experiment using a specific antibody against CSN2 for precipitation. APC1 was detected in an immunoblot using a specific anti-APC1 antibody (lanes 2–7), or as negative control, an unspecific antibody (lane 1). As a control for equal protein loading corresponding actin levels were shown.

the proteasome as well as APC/C, we performed additional protein-protein interaction assays. Hereby, we found that endogenously expressed CSN2 interacts with at least four subunits of the base of the 19S proteasome namely SUG1 [proteasome subunit p54/SUG; also named thyroid hormone receptor-interacting protein 1 (TRIP1)], TBP1 (TATA-box binding protein), 26S proteasome subunit S5B and S4. Additionally, an interaction between the COP9 subunit CSN2 and the 20S proteasome subunit alpha 6 was also detectable using the protein-protein complex identification assay (Suppl. Figs. 4–9). To further confirm these interactions, we performed a number of CoIP experiments using several antibodies that recognized different subunits of the proteasome (Suppl. Fig. 10). These results strongly suggest that endogenously expressed CSN2 is integrated in vivo in a protein complex containing the 19S sub-complex base.

Since CSN2 interacts with both the 26S proteasome and the APC/C we asked whether the COP9 signalosome is necessary to connect these two protein complexes resulting in degradation of APC/C in the proteasome. N-ethylmaleimide (NEM) is able to completely disrupt the COP9 complex.[20] In this case, a complete disruption of the COP9 complex using NEM would prevent the APC/C from binding to the proteasome. For that purpose, U2OS cell extract was preincubated with either 5 mM NEM or an equal volume ethanol as the solvent control for 1 hour followed by capturing the COP9 and 19S base containing protein complexes using specific antibodies (Fig. 3). In CoIP experiments with a SUG1-specific antibody, treatment of the cells with NEM led to a strong reduction of the co-precipitated CSN2 signal compared to untreated control cells as quantified by a densitrometric assessment (Fig. 3A; compare lane 7 with lane 3). Hereby, the co-precipitated signal from cells treated with NEM corresponding to CSN2 was reduced for approx. 75% compared to the CSN2 signal co-precipitated from control cells. This loss of interaction between CSN2 and the 19S subunit SUG1 after treatment of U2OS cells with NEM which results in the disruption of COP9 signalosome is further confirmed in similar intensity by a reverse CoIP with a specific CSN2 antibody used for co-precipitation of SUG1 (Fig. 3B). In contrast, there was only a little effect on the binding of APC1 to CSN2 or SUG1, respectively (compare Fig. 3A, lane 6 with Fig. 3B, lane 6). In summary, the binding of CSN2 to the 19S base sub-complex of the proteasome seems to be dependent on its integration in the COP9 complex. In contrast, the interaction between APC1 and CSN2 is NEM resistant which has the notion that CSN2 could bind directly to APC/C. As APC1 interacts with the base of the 19S proteasome even in the presence of NEM, we concluded that the COP9 complex is not exclusively necessary to recruit the APC/C to the proteasome.

The COP9 signalosome specifically affects stability of APC/C target proteins. In the past few years there were several reports available that COP9 influences the stability of many different targets of the ubiquitin/proteasome pathway.[21-23] This activity is mediated by different factors bound to the complex. For

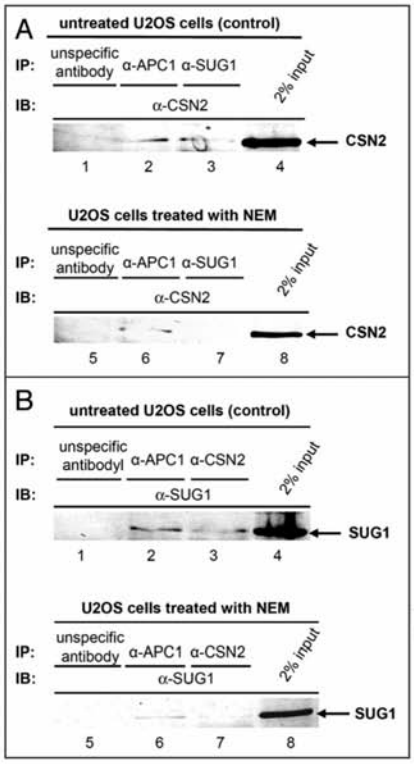

Figure 3. The interaction of CSN2 with APC1 and SUG1, respectively, is differentially affected by NEM. (A) Left panel: In CoIP experiments using crude U2OS cell extract, specific antibodies against SUG1 and APC1, respectively, co-precipitate (IP) CSN2 as detected in an immunoblot (IB) (lane 2 and 3). Right panel: The APC1 antibody was capable to co-precipitate CSN2 (lane 6) after preincubation of the U2OS cells with 5 mM NEM for one hour. In contrast, the SUG1 antibody failed to precipitate CSN2 after the mentioned treatment with NEM (lane 7). (B) Left panel: Antibodies against APC1 as well as CSN2 were both able to co-precipitate SUG1 from crude U2OS lysate (lane 2 and 3) as shown by immunoblot with a specific SUG1 antibody. right panel: A specific APC1 antibody was able to co-precipitate SUG1 from crude U2OS lysate preincubated with 5 mM NEM for one hour (lane 6). A specific CSN2 antibody failed to co-precipitate SUG1 from U2OS cells treated as described above (lane 7). An unspecific antibody which was used as negative control was not able to precipitate neither CSN2 nor SUG1 from untreated U2OS cells or treated U2OS cells (A, lane 1 and 5; B, lane 1 and 5).

example, the attached deubiquitinase USP15 mediates degradation of marked proteins.[20] For this reason, we hypothesized that the signalosome affects protein stability of APC/C targets. It was shown that overexpression of CSN2 leads to a de novo assembly of the COP9 complex.[10] Therefore, we transfected U2OS cells with an expression plasmid coding for CSN2 or, as control, the empty vector. Cells were harvested, lysed and subjected to SDS-PAGE. Thereafter, known APC/C target proteins including cyclin A, cyclin B, CDC6 and SnoN were analyzed in the differentially transfected U2OS cells by immunoblotting. In three independent experiments we detected decreased protein signals for CDC6 and SnoN in cells transfected with the CSN2 expression plasmid compared to mock-transfected control cells. In contrast, an increased signal corresponding to cyclin A was detected in cells overexpressing CSN2 due to the specific expression plasmid. No differences were found regarding cyclin B signal in specific transfected cells and control cells (Fig. 4A).

In order to proof these findings, we knocked down CSN5 by RNA interference using a specific CSN5 siRNA. As control, an unspecific non-silencing siRNA (nsc) was used. In contrast to CSN2 when a downregulation of this subunit is lethal, the CSN5 subunit of the COP9 is dispensable for complex formation but recruits several enzymes, e.g, a deubiquitinase to the signalosome.[24-26] We manipulated the activity of CSN2 and CSN5 as both subunits are located in different sub-complexes of the COP9 signalosome.[27] The COP9 is active if the complex is complete. Cells were harvested 72 h after siRNA transfection and proteins were separated on a SDS gel and analyzed by immunoblotting using specific antibodies. Signal intensities of immunoblots corresponding to CDC6, cyclin A, SnoN and cyclin B were compared between U2OS cells treated with the specific CSN5 siRNA and control cells treated with unspecific, non-silencing siRNA (Fig. 4B). Thereby, we found an increase of both SnoN and CDC6 in CSN5 downregulated U2OS cells. By contrast, there was a lower signal corresponding to cyclin A detectable in cells treated with specific CSN5 siRNA. No difference in signal intensity of cyclin B was detectable between CSN5 downregulated cells and controls. It is obvious that both CDC6 and SnoN were downregulated if a subunit of COP9 was overexpressed. In line with this both CDC6 and SnoN were up-regulated when a COP9 subunit was depleted. The results regarding cyclin A were completely opposite. The overexpression of a COP9 subunit correlates well with cyclin A upregulation well as the depletion of COP9 subunit downregulates the cyclin A expression.

Thereafter, we were interested whether the half life of the proteins was altered by transfection with a vector encoding CSN2 or, as a control, an empty vector. Cycloheximide was applied 48 h after transfection and the cells were harvested 1, 4, and 8 hours, respectively, after incubation. The protein stability of APC/C targets is influenced by CSN2 as shown in Figure 5. Both, SnoN and CDC6 were more rapidly degraded in cells over-expressing CSN2 due to a specific vector encoding CSN2 compare to control cells as densitometrical determined. A significant decreased of SnoN and CDC6, respectively, was detectable even four hours after cycloheximide induction. The half life of cyclin B was not affected by CSN2 overexpression at all. Surprisingly, we detected a decreased kinetic of cyclin A degradation in cells over-expressing CSN2 compared to control cells. These results confirm our findings regarding to the alteration of protein levels of APC/C targets after over-expression of CSN2 or down-regulation of CSN5 as

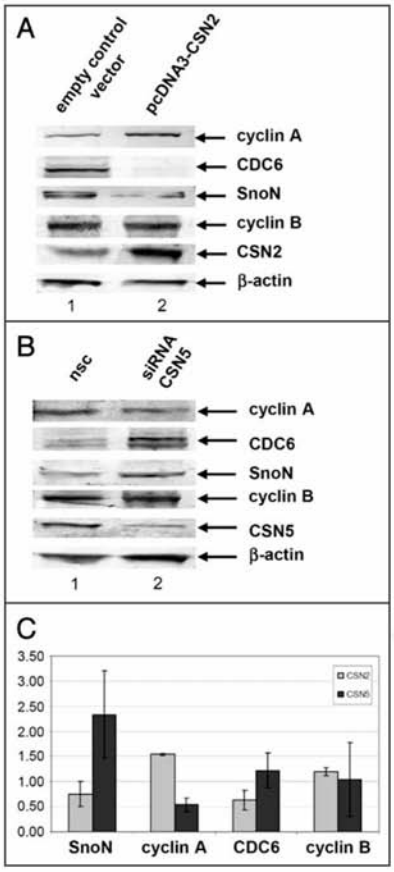

Figure 4: Overexpression of CSN2 as well as downregulation of CSN5 influenced expression of targets of the APC/C. (A) CSN2 was transiently overexpressed in U2OS cells by transfection with a pcDNA3-CSN2 plasmid (lane 2) and compared with cells transfected with the empty vector (lane 1). Transfected cells were lysed and several targets of the APC/C including cyclin A, CDC6, SnoN, and cyclin B were analyzed by immunoblot using specific antibodies. (B) Protein extracts of U2OS cells transfected with either a specific CSN5 siRNA (lane 2) or an unspecific non-silencing siRNA (nsc; lane 1) were subjected to immunoblotting against cyclin A, CDC6, SnoN, and cyclin B using specific antibodies. (C) The results of three independent experiments of CSN5 knock down and over-expression of CSN2 were densitometrically determined and summarized. The Y-axes shows the fold expression compared to controls transfected with the empty vector which were set to 1.

well as disintegration of the COP9 signalosome. Based on these findings, it seems that the COP9 signalosome influences stability of several APC/C targets on protein level.

CSN2 overexpression leads to genomic instability. Both, the COP9 signalosome and the APC/C are well characterized regulators of the cell cycle. For this reason, we asked if overexpression of CSN2 causes an altered cell cycle distribution. Fluorescence activated cell sorting (FACS)-technique was applied using U2OS cells fixed 48 h after transient transfection with the CSN2 encoding vector or, as a control, with the empty vector followed by staining of the DNA with propidium iodide and analysis of DNA content. Thereby, we did not detect any significant changes of cell cycle transition of cells expressing higher levels of CSN2 (Fig. 6A).

Consistently, there were only very slight differences in cell viability determined by colony forming assay (data not shown). Recently, it was shown that APC/C is involved in the segregation of chromatides and is necessary for accurate DNA replication.[16,28] The temporally regulated degradation of its targets seems sufficient to enable genetic stability. Since our data suggest that CDC6 protein level is altered by CSN2 overexpression, we hypothesized that this could lead to genetic instability. Using a 50K microarray analysis from Affymetrix to investigate single nucleotide polymorphism (SNP), we detected a higher rate of both deletions and duplications of genes in a pool of U2OS cells stably transfected with a vector coding CSN2 compared to a pool of control cells stably transfected with the empty vector (Fig. 6B). DNA instability was not detectable at specific sites in the genome, but they were randomly distributed over the whole genome (data not shown). These results suggest that overexpression of CSN2 leads to genetic instability and indicate that the proper regulation of the APC/C dependent protein degradation by the signalosome seems important for genetic stability. Interestingly, we did not detect an effect on the cell cycle phase distribution, although cells revealed an increase in genome instability. A possible explanation could be the lack of functional p53 in these cells. The rate of apoptosis of CSN2 over-expressing cells was analyzed by monitoring the caspase 3/7 activity. Hereby, we found only a slight reduction of the apoptotic cell fraction of CSN2 overexpressing cells compared to cells treated with the empty vector (data not shown).

Taken together, the data suggest that CSN2 interacts with the APC/C and influenced functionally the stability of APC/C targets. Further, in cells overexpressing CSN2 the genomic stability of these cells massively perturbed.

Discussion

In the past few years many publications discussed about the influence of the COP9 signalosome on the ubiquitin/proteasome pathway. It could be shown the direct interaction of Flag-CSN2 with the 26S proteasome in mouse B8 fibroblasts.[10] In the present study, we extended the panel of interacting partners of COP9 in vivo by the detection of several protein interactions between CSN2, a subunit of COP9, and subunits of the base of the 19S proteasome as well as of the 20S proteasome. These protein interactions seemingly depend on COP9 as CSN2 did not bind to the base of the 19S proteasome after disruption of the signalosome in vitro by NEM. Furthermore, we found that CSN2 binds at least three proteins of the anaphase promoting complex/cyclosome (APC/C). Beside the SKP1 Cullin F-box (SCF) complex, APC/C

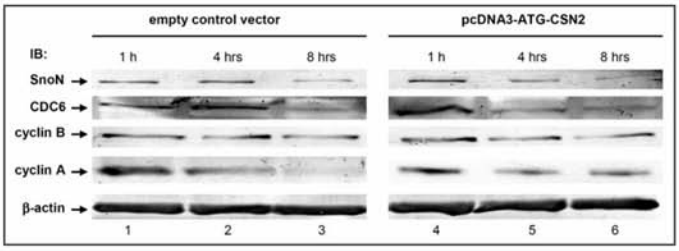

Figure 5. CSN2 deregulates stability of APC/C targets. U2OS cells were transiently transfected either with pcDNA3-CSN2 or the empty vector. 48 hours after transfection fresh medium with 50 µg cycloheximide/ml was applied and the cells were harvested after the indicated time points (1 hour: lane 1 and 4; 4 hours: lane 2 and 5; 8 hours: lane 3 and 6) and lysed using a lysis buffer. Lysates of the transfected cells treated with cycloheximide were analyzed by western blotting with antibodies specific for SnoN, Cdc6, Cyclin A and Cyclin B, respectively.

is responsible for the temporally degradation of cyclins during cell cycle.[13] As CSN2 is known to interact with cullins we suggested that it interacts directly with the cullin-like protein APC2.[24,25] Consistent with that, we found CSN2 to be bound to the APC/C even in the absence of an intact signalosome. Whether the COP9 complex recruits the APC/C to the proteasome or if these interactions are independent remains unknown. Our data clearly show that APC/C is able to bind to the proteasome even if the COP9 complex was disrupted. In our opinion, there are two possible explanations for this. On the one hand, the signalosome and the lid of the 19S proteasome compete for the APC/C mediating substrate specifity. Such a competitive binding of both complexes was shown for the binding to the base of the 19S proteasome.[10] On the other hand, it might be possible that the APC/C directly binds to the base. In this case, the COP9 would be able to influence the APC/C independently of proteasome binding.

The specificity of the APC/C for its multiple substrates is regulated by the competitive binding of Cdh1 and Cdc20 to the E3 ligase dependent on the cell cycle phase.[29-32] We found that degradation of cyclin A, SnoN and CDC6 was regulated by the COP9 complex while protein levels of cyclin B which is degraded at the end of mitosis was not affected.[33] Consistent with these data, we found that CSN2 did not bind to the APC/C during mitosis. Because of these reasons, we speculate that the regulation of APC/C by the COP9 is both high substrate and cell cycle specific.

Because of its role as a regulator of DNA duplication and chromatide separation, the APC/C was shown to mediate genomic stability.[16] Otherwise, a stabilization of APC/C targets may initiate a perturbation of these processes causing a p53 response by deregulating G_1 phase.[30] Consistently, we showed that overexpression of CSN2 and the resulting influence on the APC/C targets led to genomic alterations. This instability seems not to affect cell cycle distribution or cell viability. An explanation for unaffected cell cycle despite deregulation of APC/C targets stability might be as U2OS cell possess an inactivated form of p53. In a large genetic screen in yeast, the COP9 was shown to have a great impact on cell cycle without complete explanation for the results.[34] Our presented data show, that the signalosome regulates both the SCF and also the second important pathway for degradation of cyclins by APC/C. Additionally, it seems that COP9 is involved in regulation of the cell cycle. Taken together, CSN2 overexpression seems to lead to genomic instability. On the other hand, these cells pass cell cycle checkpoints without being arrested and are prevented from apoptosis leading to further accumulation of genomic alterations.

Materials and Methods

Cell culture and cell cycle synchronization. Human U2OS osteosarcoma cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were harvested at 70–90% confluence using PBS with 0.05% EDTA and trypsin. In order to synchronize U2OS cells, cells were submitted to different treatments as described elsewhere.[35] Mitosis: Cells were blocked in mitosis by incubation with 100 ng/ml nocodazol (Sigma) for 16–18 h. Mitotic cells were detached by mitotic shake-off and cleared from the medium by centrifugation for 5 min on 1500 rpm at room temperature. G_1 phase: Synchronization in G_1 was achieved by mimosine treatment (0.5 mM, for 24 h, Sigma). G_1/S phase: to accumulate cells at the G_1/S transition hydroxy urea (Sigma; 2 mM, overnight) was used. S phase/G_2 phase: In a first step, U2OS cells were blocked in G1 by serum starvation for 24 h. Afterwards fresh medium with FBS supplemented with thymidine (Sigma; 2.5 mM over night) was added. The cell cycle block was released by washing cells twice with PBS and normal medium was applied. Cells were harvested after 4 h reflecting to S phase or after 8 h corresponding to G_2 phase, respectively.

Cells were lysed in a buffer containing 100 mM sodium phosphate pH 7.5, 5 mM EDTA, 2 mM MgCl2, 0.1% CHAPS, 500 µM leupeptin, and 0.1 mM PMSF. After centrifugation (15 min; 15,000 rpm; 4°C) the supernatant was immediately used.

Protein-protein complex identification assay. The protein-protein interaction assay was performed as described before.[36] In short, 4 µl protein A (Sigma) was bound to 20 µl of Interaction Discovery Mapping (IDM) beads (Bio-Rad) overnight at 4°C. After discarding the supernatant the pellet was washed twice with a buffer containing 50 mM sodium acetate pH 5.0. Afterwards, unspecific binding sites were blocked by incubation with a buffer

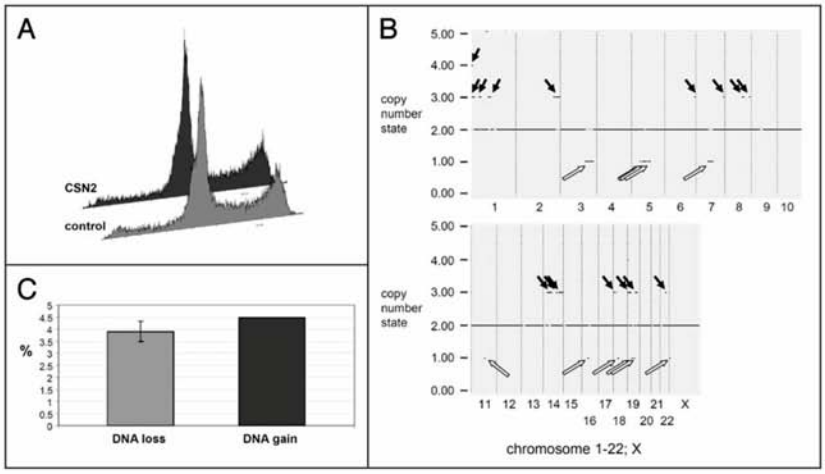

Figure 6. CSN2 overexpression promotes genetic instability. (A) U2OS cells were transfected with pcDNA3-CSN2 or the empty vector and ethanol fixation following staining of DNA with propidium iodide and measuring cell cycle distribution by FACS analysis. (B) Stably transfected U2OS cells with pcDNA3-CSN2 or the empty vector, respectively, were analyzed with a 50K microarray (Affymetrix), which shows the copy number of genomic sequences. CSN2 overexpressing cells exhibit loss as well as well as gain of allels compared to cells transfected with the empty vector control. The DNA changes in the control cells transfected with the empty vector were normalized to a copy number of 2 chromosomes. Dots above the black line imply DNA amplifications in cells transfected with the pcDNA3-CSN2 vector (copy number of 3; black arrow); dots below the black line shows DNA losses in CSN2 over-expressing cells (copy number 1; white arrow). The experiment was done twice. (C) Percentage DNA loss or DNA gain, respectively, of the whole genome of the CSN2 over-expressing cells.

containing 0.5 M Tris/HCl pH 9.0 and 0.1 % Triton X-100 for 1 hour at room temperature. The beads were washed three times with 1x PBS. Thereafter, a specific antibody which recognized human CSN2 (rabbit polyclonal), or normal rabbit IgG (Pepro Tech Inc.; Rocky Hill, NJ) as negative control, in 50 mM sodium acetate pH 5.0 was applied to the beads and allowed to bind at room temperature for 1 hour in an end-over-end mixer. The specific anti-CSN2 antibody was described before.[37] Unbound antibodies were removed by washing in 50 mM sodium acetate. Following two washes with 1 x PBS the beads were incubated with at least 100 µl of crude U2OS cell extract for two hours at 4°C in an end-over-end mixer. Unbound proteins were removed by sequential washes in 0.5 M sodium chloride, 0.1 % Triton X-100 and PBS. Afterwards proteins were eluted from the IDM beads by 25 µl 50% acetonitrile/0.5% trifluoroacetic acid and gently vortexed for 30 minutes. Five microliters of the eluted samples were subjected on an activated H50 ProteinChip Array (Ciphergen Biosystem, Inc., Fremont, CA) and the array was analyzed in a ProteinChip Reader (series 4000; Ciphergen, Bio-Rad) according to an automated data collection protocol by SELDI-MS. This includes an average of 265 laser shots to each spot with a laser intensity of 2300 nJ and 3500 nJ (20–200 kDa), respectively, dependent on the measured region (low = 2.5–20 kDa and high = 20–200 kDa, respectively) and an automatically adapted detector sensitivity.

The volume of the eluted samples was reduced to a maximum of 10 µl using a speed-vac (ThermoServant) and subjected to SDS-PAGE for separation of containing proteins followed by staining with Simply Blue Safe Stain (Enhanced Coomassie, Invitrogen). Specific gel bands were excised, destained, and dried followed by rehydration and digestion with 10 µl of a trypsin solution (0.02 µg/µl; Promega) at 37°C overnight. The supernatants of the in-gel digestions were applied directly to a gold (Au) arrays (Bio-Rad). After addition of the matrix (CHCA), peptide fragment masses were analyzed by SELDI-MS. A standard protein mix (all-in-1 peptide standard mix; Bio-Rad), including Arg8-vasopressin (1082.2 Da), somatostatin (1637.9 Da), dynorphin (2147.5 Da), ACTH (2933.5 Da), and insulin beta-chain (3495.94 Da) was used for calibration. Proteins were identified using the fragment masses searching in a publicly available database (Profound; prowl.rockefeller.edu/prowl-cgi/profound.exe).

Co-immunoprecipitation. Specific antibodies which recognise CSN2 (rabbit polyclonal), S4 (rabbit polyclonal; Abcam), SUG1 (25D5, mouse monoclonal; Abcam), p42 (p42-23, mouse monoclonal; Calbiochem), TBP1 (TBP1-19, mouse monoclonal), TBP7 (TBP7-27, mouse monoclonal), proteasome α 6 subunit (MCP20, mouse monoclonal; Abcam), APC1 (H-300, rabbit polyclonal; Santa Cruz) or, as negative control, normal rabbit IgG (Pepro Tech Inc.) were bound on protein A-agarose beads. The antibody loaded beads were incubated with 150 µl of crude U2OS cells extract for 1 hour at 4°C. Unspecific bound proteins were removed by three washes with CoIP buffer containing 20 mM HEPES/KOH pH 8.0, 50 mM KCl, 0.1 mM EDTA and 0.05% CHAPS.

Afterwards beads were boiled in 4 x SDS loading buffer (200 mM Tris-Cl pH 6.8, 4% SDS, 30% Glycerol, 10% β-mercaptoethanol, 0.002% Bromophenol blue) and bound proteins were subjected to a 10% SDS–PAGE and analyzed by immunoblotting. For detection in immunoblots following antibodies apart from the ones mentioned above were used: CSN2 (goat polyclonal; Abcam), β-actin (A266, rabbit polyclonal; Sigma), cyclin A (C-19, rabbit polyclonal; Santa Cruz), cyclin B1 (M-20, rabbit polyclonal; Santa Cruz), CDC6 (0.T.17, mouse monoclonal; Santa Cruz), SnoN (H-317, rabbit polyclonal; Santa Cruz) and CSN5 (FL-334, rabbit polyclonal; Abcam).

For CoIP experiments using crude U2OS cell extract preincubated with N-ethylmaleimide (NEM), lysates were split into two aliquots. The sample was treated either with 5 mM NEM or with an equal volume of ethanol at 4°C for 1h in an end-over-end mixer.

Small interfering RNA-mediated knockdown of CSN5. For knockdown by RNA interference the following small interfering RNA (siRNA) duplex oligonucleotides was used in this study that was based on the human cDNA encoding CSN5. CSN5: 5'-GCAAUCGGGUGGUAUCAUAdTdT-3'(sense), 5'-UAUGAUACCCGAUUGCdAdT-3' (antisense) (QIAGEN GmbH, Hilden, Germany); nonsilencing control siRNA: 5'-UUCUCCGAACGUGUCACGUdTdT-3' (sense), 5'-ACGUGACACGUUCGGAGAAdTdT-3' (antisense) (QIAGEN GmbH, Hilden, Germany). U2OS cells (3 x 10^5) were seeded in a six-well plate 24–48 h before transfection and were 50% confluent when siRNA was added. The amount of siRNA duplexes applied was 1.5 µg/well for CSN5. Transfection was performed using the amphiphilic delivery system SAINT-RED (Synvolux Therapeutics B.V., Groningen, The Netherlands) as described.[38] Briefly, siRNA was complexed with 15 nmol of transfection reagent and added to the cells for 4 hours. Subsequently, 2 ml of culture medium was added and incubation proceeded for 72 h.

Transfection. U2OS cells were seeded into 6-well plates at a density of 2.5 x 10^5 cells per well 24 h prior to transfection. Fresh DMEM supplemented with 10% FCS was added four hours before transfection. Afterwards, transfection was performed with $CaPO_4$ as described.[22] Hereby, one microgram of pcDNA3-Linker or pcDNA3-CSN2 were used per well. Cells were washed three times with 2 ml PBS and new medium was applied after 24 h.

In case of transient transfection, cells were harvested 48 h after initial transfection. In case of cycloheximide chase, 50 µg cycloheximide per ml medium were applied and cells were collected after 1, 4, and 8 hours, respectively. For stable transfection, cells were selected with 0.5 mg/ml geneticin sulphate (G418). Medium and hygromycin were replaced every 2–3 days until all cells were died in a transfection control experiment. All experiments were performed at least three times.

Densitometrical assessment. Signal intensities of corresponding proteins were densitrometrical assessed using the Lab Image 1D program (Kapelan Bio-Imaging, Leipzig, Germany) according to the manufacturer's instructions.

FACS. Cell cycle distribution of U2OS cells were shown by fluorescence activated cell sorting (FACS) as described.[39] Harvested transiently transfected cells and medium were collected together to obtain all cell cycle phases and apoptotic cell population. After centrifugation (5 min, 1500 rpm, RT) the resulting pellet was washed twice in PBS. For fixation, 1 ml of ice-cold 70% ethanol was added slowly to the cells. Afterwards, the cells were resuspended carefully and incubated for 1 h on ice. After centrifugation and washing in PBS, the cells were resuspended in 300 µl PBS with 1 mg/ml RNase A (Fermentas). The samples were incubated for 10 min at room temperature before staining the DNA with 50 µg propidium iodide (Sigma) for 10 min at RT. Fluorescent labeling was measured with a FAC-Scan using Cell Quest Software (Becton Dickinson).

Apoptosis assay. Changes of the apoptotic population of U2OS cells transiently transfected with either pcDNA3-CSN2 or the empty vector, respectively, were analyzed with the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) according to manufacturer´s instructions. Hereby, the amount of total protein of cell lysates was measured with a NanoDrop device (ND-1000 spectrometer; Peqlab, Erlangen, Germany) and samples were diluted to same concentrations. Afterwards, samples were measured on a fluorescence plate reader with excitation maximum at 498 nm and emission maximum of 521 nm.

Genetic stability. The gene chip copy number analysis was based on the detection of single nucleotide polymorphisms (SNP). Therefore, a 50K microarray from Affymetrix was used to detect differences of chromosomal imbalance between U2OS cells overexpressing CSN2 and untreated U2OS cells. The procedure was performed according to the Mapping 100K Assay manual from Affymetrix (www.affymetrix.com). First, total DNA was isolated from U2OS cells using a Qiagen Mini Kit following by digestion with XbaI restriction enzyme and amplification by one-primer PCR. After amplification the genomic DNA was purified, fragmented and labeled. Finally, the DNA fragments were hybridized to the 50.000 SNPs on the XbaI microarray surface. After 16 h hybridization, the DNA was stained with streptavidin phycoerythrin (SAPE), washed and scanned. The difference in fluorescence intensity was caused by variation in concentration of bound DNA. This difference indicates a loss or gain of chromosomal material. Following primers were used. XbaI adaptor sequence: 3'-AATACTCGTGCTGTCTGCGGACTAGAGATCT-5'; PCR primer, 001: 5'-ATTATGAGCACGACAGACGCCTGATCT-3'.

Acknowledgments

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/KobCC8-13-Sup.pdf

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2.4 The COP9 regulation of the APC/C is modulated by Alienα

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In dieser Studie wurde gezeigt, dass neben CSN2 auch Alienα mit dem APC/C funktionell interagieren kann. Dabei verändert Alienα die Degradationskinetik einiger APC/C-Targetproteine und kann durch Akkumulation mitotischer Cycline in der G1-Phase zu einer erhöhten genetischen Instabilität führen. Darüber hinaus beeinflusst Alienα die Stabilität sowohl des APC/C-Komplexes als auch einiger seiner Untereinheiten. Anhand dieser Daten zeigt sich das komplexe Zusammenspiel der beiden Alien-Isoformen in der Regulation der proteasomalen Degradation.

Bis auf den Phosphorylierungsassay (Dr. Stephan Tenbaum) und die Analyse der genetische Instabilität (Nicole Posorski) wurden alle experimentellen Laborarbeiten von mir durchgeführt. Für diese Arbeit wurden Plasmide von Dr. Christian Hoischen (APC2-Domänen) und Prof. Aria Baniahmad (Alien-Isoformen) bereitgestellt.

The COP9 regulation of the APC/C is modulated by Alienα

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Running title: Alienα is involved in regulation of APC/C

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In the last few years the COP9 signalosome was described to regulate the two key players of cyclin degradation – the Skp1/cullin/F-box complex and the anaphase promoting complex/cyclosome (APC/C). CSN2/Alienβ, which is the COP9 subunit directly involved to facilitate these interactions has an isoform called Alienα. Here we show that Alienα is also able to interact with the APC/C in vitro as well as in vivo. CSN2/Alienβ and Alienα compete for binding to the APC/C resulting in altered degradation kinetics of several APC/C target proteins. Mutation of the potential CDC2 phosphorylation site at Ser270 of Alienα disrupts the interaction between Alienα and APC/C. Mutation of this phosphorylation site causes accumulation of mitotic cyclins in G1 resulting in a faster cell cycle and higher genetic instability. Interestingly, an Alienα mutant that mimics a constitutive phosphorylation at Ser270 disrupts the APC/C and several of its subcomplexes. This phospho mutant of Alienα was able to permit a slow cell cycle and even reduced the number of apoptotic cells. Taken together, these data suggest Alienα as a regulator of the APC/C with a high impact on cell proliferation and genetic stability.

A tight regulation of the degradation of damaged or misfolded as well as short-lived proteins is necessary to maintain a stable cell cycle. Two multisubunit E3 ubiquitin ligase complexes named APC/C (anaphase promoting complex/cyclosome) and SCF (Skp1/Cullin/F-box) are the main components, which mark cell cycle regulators with ubiquitin (Ub) for destruction by the 26S proteasome [1, 2]. During the last years the COP9 signalosome was described as a modulator of the SCF [3]. T9 signalosome (also named CSN) was first identified in Arabidopsis and comprises eight conserved subunits, CSN1-CSN8, in all eukaryotic cells [4]. The signalosome exhibits a high homology to the 19S lid of the 26S proteasome as well as to the translational initiation complex eIF3 [5, 6].

Data suggest a competition between the COP9 complex and the 19S lid for binding to the 20S proteasome [7, 8]. It was shown that CSN1 interacts with Rpn6, a subunit of the 19S lid in both Arabidopsis as well as in Drosophila [9, 10]. Additionally, subunits of COP9 were found to interact with different subunits of cullin-dependent E3 ligases. Furthermore, the COP9 complex seems to recruit E3 ligases to the 26S proteasome forming supercomplexes [11]. Especially, the metalloproteinase activity of CSN5, which cleaves Nedd8 from cullins is necessary for the regulation of the SCF complex [12]. Not he ubiquitinating activity but also the stability of the cullin

containing E3 ligases are influenced by cycles of neddylation and deneddylation [13]. In a very recent study we revealed an interaction of the COP9 complex with the APC/C [14]. Furthermore, we found that the signalosome is able to regulate APC/C, the second main mediator of cyclin degradation. Since APC/C promotes the ongoing of the cell cycle, especially controls the segregation of centrosomes it seems that APC/C is involved in the maintenance of the genomic stability [2, 15, 16]. Consistent with these findings, the COP9 is essential for cell cycle progression and genomic stability in Arabidopsis [17].

The CSN2 isoform Alienα represents the N-terminal 305 amino acids of the CSN2/Alienβ subunit of COP9 signalosome [18]. The PCI domain which is important for the assembly of the COP9 complex is absent in Alienα [19]. In line with this, only CSN2/Alienβ but not its N-terminal Alienα isoform is integrated into the signalosome [7]. On the other hand, the N-terminus of CSN2 interacts with cullins [20, 21]. Consequently, Alienα could potentially bind to APC2. Based on these observations, it was the aim of the present study to analyze the influence of Alienα on the functional interaction between the APC/C and the signalosome. Furthermore, we asked whether a possible interference of the interplay between COP9 and APC/C by Alienα leads to alterations of the cell cycle and cell proliferation that potentially results in genomic instability and tumor development.

Experimental Procedures

Antibodies: For detection in immunoblots, the following specific antibodies were used: β-actin (A266, rabbit polyclonal; Sigma), Alien (rabbit polyclonal [22]), APC1 (H-300, rabbit polyclonal; Santa Cruz), APC2 (Ab-1, rabbit polyclonal; MeoMarkers), Cyclin A (C-19, rabbit polyclonal; Santa Cruz), Cyclin B1 (M-20, rabbit polyclonal; Santa Cruz), CDC6 (0.T.17, mouse monoclonal; Santa Cruz), CDC27 (C-4, mouse monoclonal; Santa Cruz), CSN2/Alienβ (goat polyclonal; Abcam) and SnoN (H-317, rabbit polyclonal; Santa Cruz).

Cell culture and cell cycle synchronization: Human U2OS osteosarcoma cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were harvested at 70-90% confluence using PBS with 0.05% EDTA and trypsin. The cell synchronization protocol is described elsewhere [23]. Mitosis: Cells were blocked in mitosis by incubation with 100 ng/ml nocodazol (Sigma) for 16–18 h. Mitotic cells were detached by mitotic shake-off and cleared from the medium by centrifugation for 5 minutes on 1500 rpm at room temperature. G1 phase: Synchronization in G1 was achieved by mimosine treatment (0.5 mM, for 24 hours, Sigma). G1/S phase: to accumulate cells at the G1/S transition hydroxy urea (Sigma; 2 mM, over night) was used. S phase/G2 phase: In a first step U2OS cells were blocked in G1 by serum starvation for 24 hours. Afterwards fresh medium with FBS supplemented with thymidine (Sigma; 2.5 mM over night) was added. The cell cycle block was released by washing twice with PBS and normal medium was applied. Cells were harvested after 4 hours for S phase or after 8 hours corresponding to G2 phase, respectively.
Cells were lysed in a buffer containing 100 mM sodium phosphate pH 7.5, 5 mM EDTA, 2 mM $MgCl_2$, 0.1% CHAPS, 500 µM leupeptin, and 0.1 mM PMSF. After centrifugation (15 minutes; 15000 rpm; 4°C) the supernatant was used immediately.

Pulldown assays: His-tag-pulldown experiments were performed as described before (Evan et al., 1985). Briefly, His-APC2 (aa 1-271), His-APC2 (aa 318-730) or His-APC2 (aa 745-822) were bacterially expressed in the E.coli strain BL21(lys) via induction of the tac-promoter by adding 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture medium and incubation for 3 hours at 37°C. The cells were harvested by spinning for 5 minutes at 6,000 rpm, resuspended in sonication buffer (50 mM $NaPO_4$ pH 7.8; 300 mM NaCl) and lysed by freezing in liquid nitrogen and sonification after thawing. The APC2 domains were purified using a Ni-cellulose resin (Amersham Biosciences) and residual bound proteins were eliminated by four washes with wash buffer (50 mM $NaPO_4$ pH 6.0; 300 mM NaCl; 10% glycerol). Remaining binding sites were blocked with 3% BSA solution. Interaction studies were performed with in vitro translated

(S30 T7 High-yield protein expression system, Promega Corp.) Alienα wt, Alienα Ser270Ala, Alienα Ser270Asp, respectively. Equal amounts of the Alienα isoforms were incubated with the beads. After several washes with wash buffer, bound proteins were separated by SDS-PAGE and detected by Western blotting using a specific anti-Alienα antibody (PepAK2).

Co-immunoprecipitation: Specific antibodies that recognize Alien (rabbit polyclonal [22]), APC1 (H-300, rabbit polyclonal; Santa Cruz) or, as negative control, normal rabbit IgG (Pepro Tech Inc.) were bound to protein A-agarose beads. The antibody loaded beads were incubated with 150 µl of crude U2OS cells extract for 1 hour at 4°C. For binding studies, dependent on the cell cycle phase, we used lysates of synchronized cells as described above. Competition assays were performed by adding bacterially expressed Alienα, CSN2/Alienβ or both, in equal concentrations. In additional assays, increasing amounts (10 ng, 100 ng or 1000 ng respectively) of in vitro translated Alienα were employed. Unspecific bound proteins were removed by three washes with CoIP buffer containing 20 mM HEPES/KOH pH 8.0, 50 mM KCl, 0.1 mM EDTA and 0.05% CHAPS. Afterwards beads were boiled in SDS loading buffer (200 mM Tris-Cl pH 6.8, 4% SDS, 30% Glycerol, 10% β-mercaptoethanol, 0,002% Bromophenol blue) and bound proteins were subjected to a 10% SDS–PAGE and analyzed by immunoblotting.

Transfection: U2OS cells were seeded into 6 well plates at a density of 1.5 x 10^5 cells per well 4 hours prior to transfection. Afterwards, transfection was performed with $CaPO_4$ as described [24]. Hereby, one microgram of pcDNA3-Linker, pcDNA3-Alienα wt, pcDNA3-Alienα Ser270Ala, pcDNA3-Alienα Ser270Asp or pcDNA3-CSN2/Alienβ were used per well. After 16 hours cells were washed two times with 2 ml PBS and new medium was applied.
In case of transient transfection experiments, cells were harvested 36 hours after initial transfection. For cycloheximide chase, 50 µg of cycloheximide per ml medium was applied and cells were collected after the indicated times, respectively. When degradation of mitotic cyclins was monitored cells were blocked in mitosis with 100 ng/ml nocodazol overnight, relased by addition of fresh medium and harvested after 0, 1, 2 or 3 hours, respectively. For stable transfection, cells were selected with 1 mg/ml geneticin sulphate (G418). Medium and hygromycine were replaced every 2-3 days until all cells in an untransfected control died. All experiments were performed at least three times.

Phosphorylation assays: For in vivo phosphorylation of Alien proteins equal amounts of HA-tagged Alien expression plasmids (pHA-Alienα) and empty HA-vector (pHA-linker) were transfected into HEK293 cells. The next day the medium was changed to phosphate and serum-free medium and the cells were incubated over night. The next day 0.6 mCi ^{32}P-ortho-phosphate was added to each dish and the cells were incubated again for 5 hours. Thereafter, the cells were washed twice in PBS and lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5mM $CaCl_2$, 5 mM $MgCl_2$, 1% NP-40, 1% Triton X-100, 1 mM PMSF, 10 ng/ml leupeptin, 10 ng/ml aprotinin). Then the HA-tagged Alien was immunoprecipitated using a specific HA antibody. Samples were separated by SDS-PAGE, the gel was dried and the radioactively labeled proteins were visualized by autoradiography. In case of the in vitro kinase assays HeLa cells were lysed with 400 µl CDC2-lysis buffer (20 mM Tris-HCl pH 7.4; 100 mM NaCl; 1% Triton X-100; 10 mM EGTA; 10 mM EDTA; 40 mM β-glycero phosphate; 2 mM orto-vanadate; 1 mM PMSF; 10 ng/ml leupeptin and 10 ng/ml aprotinin). Immunoprecipitations of the Cdk1 kinase complex were performed with anti-Cyclin A or anti-Cyclin B antibody (kindly provided by C. Cales, IIB, Madrid, Spain), respectively. The kinase reaction (30 minutes, 37 ºC) was carried out in presence of 50 µM non-radioactive ATP and with 7.4 x 10^4 Bq ^{32}P-γATP in the kinase assay mix (25 mM Tris/HCl pH 7.5; 10 mM $MgCl_2$; 1 mM DTT). An additional purification step was performed in these assays. After the kinase reaction, the reaction mixtures were filled up to 150 µl with CDC2-kinase assay mix, mixed well, centrifuged and the supernatant was transferred to new tubes. This step was repeated 3 times and the supernatants were combined. Then the GST-fused substrates were re-purified from these solutions using 20 µl glutathione Sepharose 4B analog to GST-

pulldown assays rocking at room temperature for 30 min. The glutathione Sepharose beads were washed 3 times with PBS and were denatured for 3 minutes at 100ºC in SDS loading buffer. CDC2-kinase reactions were separated with 10% SDS-PAGE gels, dried and exposed to X-ray films.

Blue native PAGE: Blue native PAGE (BN-PAGE) was performed using the NativePage™ Novex® Bis-Tris gel system (Invitrogen). Briefly, stable transfected cells were lysed in NativePAGE sample buffer supplemented with 1% n-dodecyl-β-D maltoside (DDM). After centrifugation (15 minutes; 15000 rpm; 4°C) the protein concentration of the supernatant was measured with a NanoDrop® spectrophotometer (ND-1000 spectrometer; Peqlab, Erlangen, Germany). NativePAGE G-250 sample additive was added at a final concentration of 0,5%. After separation of the complexes on a 4-16% gel the proteins were blotted on a PVDF membrane. Residual Coomassie was washed away with methanol and the complexes were detected with specific antibodies directed against APC1, APC2 and CDC27. To verify equal protein loading, a part of the lysate was separated by a denaturing SDS-PAGE and the amount of β-actin was analyzed by immunoblotting.

Quantitative real-time PCR: Total RNA was isolated using TriReagent (Sigma) according to manufacturer's instructions. Two µg of total RNA were used for cDNA synthesis with a mixture of random/ oligo-dT primers and the Superscript™ First Strand Synthesis system (Invitrogen). Real time PCR for relative quantification of *CDC27* expression levels was performed using LightCycler instrument (Roche) and Quantitect SYBR Green Kit (Qiagen). Sequences of used *CDC27* oligos were forward: 5' CCA AAT Tgg AAg ggC CTA TT 3', reverse: 5' TAT CAT gTT CCC gTT gCA gA 3'. Amplification of ß-actin as an endogenous control was used to standardize the amount of sample added to the reactions (forward: 5'ACA gAg CCT CgC CTT TgC CgA 3', reverse: 5' CAC gAT ggA ggg gAA gAC g 3'). The following PCR protocol was used: pre-incubation: 15 minutes at 95°C, amplification: 10 seconds at 95°C, 10 seconds at 58°C, 20°C at 72°C for 40 cycles, melting curve: 96°C/ 65°C/ 95°C, slope 0.1°C/s with continuous data acquisition.

Colony forming assay: U2OS cells stably transfected with either pcDNA3-Linker, pcDNA3-Alienα wt, pcDNA3-Alienα Ser270Ala or pcDNA3-Alienα Ser270Asp were harvested and counted with a CASY® 1 (Schaerfe System). 1000 cells were replated in 6 well plates and cultured for about 14 days under selection with 1 mg/ml geneticin sulphate until colonies with ~30 cells have formed in the control. Colonies were then washed once with PBS, fixed with methanol for 15 minutes, stained with Giemsa dye, and finally air-dried. The number of colonies formed was then determined.

FACS: Cell cycle distribution of U2OS cells were shown by fluorescence activated cell sorting (FACS). Transiently transfected cells were harvested and collected together with the medium to obtain all cell cycle phases and apoptotic cell population. After centrifugation (5 minutes, 1500 rpm, RT) the resulting pellet was washed twice with PBS. For fixation, 1 ml of ice-cold 70% ethanol was added slowly to the cells. Afterwards, the cells were resuspended carefully and incubated for 1 hour on ice. After centrifugation and washing in PBS, the cells were resuspended in 300 µl PBS with 1 mg/ml RNase A (Fermentas). The samples were incubated for 10 minutes at room temperature before staining the DNA with 50 µg propidium iodide (Sigma) for 10 minutes at RT. Fluorescent labeling was measured with a FAC-Scan using Cell Quest Software (Becton Dickinson).

Apoptosis assay: Changes of the apoptotic population of U2OS cells transiently transfected with either pcDNA3-CSN2/Alienβ or the empty vector, respectively, were analyzed with the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) according to manufacturer´s instructions. Hereby, the amount of total protein of cell lysates was measured with a NanoDrop device and samples were diluted to the same concentrations. Afterwards, samples were measured on a fluorescence plate reader with an excitation maximum 498 nm and an emission maximum at 521 nm.

Genetic stability: To investigate chromosomal imbalances in U2OS cells after stable transfection

with either pcDNA3-Linker, pcDNA3-Alienα wt, pcDNA3-Alienα Ser270Ala or pcDNA3-Alienα Ser270Asp the high resolution array-comparative genomic hybridization (CGH) platform from Agilent (Agilent technologies, USA) was used. Array-CGH experiments were performed with the Human Genome Microarray Kit 105A according to the manufacturer's instructions (www.agilent.com, protocol vs. 5.0, June 2007). The genomic DNA (gDNA) was isolated from the cells about 4 weeks after transfection using the QIAmp DNA Mini Kit from Qiagen. The gDNA of cells transfected with pcDNA3-Linker was used as reference. 1 µg of test and reference DNA was digested with restriction enzymes AluI and RsaI. Then both DNA samples were labeled with a fluorescent dye: Cy5 (test) and Cy3 (reference) with the Agilent Genomic DNA Enzymatic Labelling Kit Plus. Labelled test and reference DNA were purified, measured, combined, denatured, pre-annealed with both Cot-1 DNA (Roche) and blocking reagent (Agilent) and then hybridized to the 60mer oligonucleotides on the 105K microarray-surface. After 40 hours of hybridization in a rotating oven (Agilent Technologies, USA) at 65°C and 20 rpm the microarray was washed and scanned at 5 µm resolution with an Agilent G2565CA scanner. The raw data (tiff-Images) were extracted with Feature Extraction Software 10.5.1.1. (Agilent Technologies, USA) and analyzed with CGH Analytics Software 3.5.14. (Agilent Technologies, USA).

Results

Alienα competes with CSN2/Alienβ for binding to the APC/C. Very recently, we showed that CSN2/Alienβ interacts with APC/C in vivo [14]. CSN2/Alienβ interacts with Cullin 1 through a site within its N-terminal 189 aa region [21]. For this reason, we hypothesized that Alienα, which consists of the first 305 aa of CSN2/Alienβ is also able to interact with the cullin-like APC2. To test this hypothesis, we performed pulldown experiments. Full length APC2 is too large for bacterial expression. The domain structure of APC2 was analysed using DomPro, SBASE and SMART (www.ics.uci.edu/~baldig/dompro.html, hydra.icgeb.trieste.it/sbase/, smart.embl-heidelberg.de) and resulted roughly in a tripartite structure, an N-terminal domain 1 with unclear function, a cullin like domain 2, and a C terminal APC2 specific domain 3. We subdivided APC2 and cloned the domain 1 containing region (aas 1 – 271), the domain 2 containing region (aas 318 – 730), and the domain 3 containing region (aas 745 – 822) as his-tagged fragments into bacterial expression vectors. Thereafter, each of the APC2 domains was bound to nickel cellulose beads and in vitro translated Alienα was added. As negative control we used nickel cellulose beads without bound proteins. After several washes the proteins were eluted and separated by SDS gels and Alienα was detected in an immunoblot. Here, we found that Alienα interacts with the cullin domain of APC2 (domain 2) with about ten fold higher intensity compared to the other two APC2 domains. The negative control did not show a signal corresponding to Alienα (Fig. 1A).

Since CSN2/Alienβ contains the same potential binding site for interaction with APC/C as Alienα we were interested whether the two isoforms compete for binding to this complex. For further analysis the APC/C subunit APC1 was chosen to exclude that Alienα only binds to APC2 but not to the APC/C complex. In order to proof our hypothesis we carried out competing co-immunoprecipitation assays to co-precipitate CSN2/Alienβ or Alienα, respectively, by a specific APC1 antibody (Fig. 1B). Hereby, we added bacterial expressed Alienα and/or CSN2/Alienβ to U2OS cell extract in precipitation assays. Apparently, Alienα abolished binding of endogenous CSN2/Alienβ to endogenous APC1. In line with this, the precipitation of CSN2/Alienβ by a specific APC1 antibody was more effective when CSN2/Alienβ was added without Alienα to the cell extract. Thus, it seems that Alienα competes with CSN2/Alienβ for binding to the APC/C and decreases the APC1-CSN2/Alienβ interaction.

In a next step we asked if the competition by Alienα leads to a stepwise decline of the APC1-CSN2/Alienβ interaction. Therefore, we added increasing amounts of in vitro translated Alienα in co-immunoprecipitation assays from U2OS cell extract. Obviously, a slight addition of Alienα facilitated a more effective binding of endogenous CSN2/Alienβ to endogenous APC1. This effect

was abrogated by higher concentrations of Alienα confirming a competition (Fig. 1C).

Alienα alters degradation of several APC/C targets. Since Alienα affects the interaction of CSN2/Alienβ with the APC/C we hypothesized that Alienα influences the degradation of APC/C target proteins. To assess this assumption, we first analyzed the protein levels of several endogenous APC/C targets in U2OS cells transfected with an Alienα or a CSN2/Alienβ expression vector by western blotting (Fig. 2A). Hereby, CSN2/Alienβ and Alienα induced opposite effects on the cellular levels of Cyclin A, CDC6 and SnoN. The levels of Cyclin A were lowered by Alienα, those of CDC6 and SnoN were stabilized. The protein level of Cyclin B was altered by neither of the isoforms (Fig. 2A). Results of three independent immunoblot experiments were densitometrically analyzed and summarized (Fig. 2B).
Next, we examined if the effect on the protein levels was caused by changes in the degradation kinetics. It was shown that SnoN degradation was altered by CSN2 [14]. Furthermore SnoN is known to be targeted for degradation only by the APC/C it was used for cycloheximide chase experiments [25, 26]. Hereby, we detected signals in immunoblot experiments corresponding to SnoN that showed stable intensity at all investigated time points in cells containing transiently overexpessed Alienα compared to SnoN signals analyzed in control cells treated with an empty expression vector. These results suggest that in U2OS cells overexpressing Alienα degradation of SnoN is inhibited (Fig. 2C). The result of the cycloheximide chase experiment was densitrometicaly analyzed.
Thus Alienα influences the degradation kinetics of APC/C targets. This implies the question if the cell proliferation is influenced by the Alien isoforms. Therefore, colony forming assays were performed. The stable overexpression of Alienα leads to a decrease in cell proliferation capacity whereas stably overexpressed CSN2/Alienβ showed no differences compared to cells stably transfected with the empty vector (Fig. 2D). Cells transiently overexpressing CSN2/Alienβ exhibit a lowered apoptosis rate compared to cells overexpressing Alienα (Fig. 2E). Taken together, Alienα influences protein levels of APC/C degradation targets and inhibits cell proliferation without signs of apoptosis.

The interaction of Alienα with APC1 depends on the cell cycle phase. -- Since CSN2/Alienβ interacts with the APC/C in a cell cycle dependent manner we hypothesized that Alienα might act similarly [14]. For that purpose, U2OS cells were synchronized at different cell cycle phases and co-immunoprecipitation experiments with the extracts were performed using specific antibody against APC1. Afterwards, the presence of co-precipitated endogenous Alienα was analyzed by western bloting (Fig. 3A). The data suggest that a cell cycle phase dependent interaction between APC1 and Alienα exists. The APC1 antibody co-precipitated decreased amounts of Alienα in G1 compared to the G1/S transition or the S and G2 phases. In mitotic cells, there was only a weak Alienα/APC1 interaction detectable (Fig 3A). In conclusion, the Alienα/APC1 interaction seems to be dependent on the different cell cycle phases and it is disturbed in mitosis.
In a next step we were interested in the regulation of this specific interaction. So the question was addressed if Alienα could be phosphorylated. Therefore, an in vivo labeling assay using ^{32}P-ortho-phosphate in HEK293 cells was carried out. The cells were transfected with expression plasmids coding for hemaglutinin-tag (HA) alone, HA-Alienα or HA-Alienα fusions. After the in vivo labeling with 7.4 x 10^4 Bq ^{32}P-ortho-phosphate per dish HA, HA-Alienα and HA-Alienβ were immunoprecipitated using specific anti-HA antibody, separated by SDS-PAGE and visualized by autoradiography. This autoradiography shows that HA-Alienα was strongly labeled with ^{32}P (Fig 3B). Potential cell cycle dependent phosphorylation sites of Alienα were predicted by a public online database (http://csbl.bmb.uga.edu/~ffzhou/gps_web/predict.php). Hereby, the serine 270 residue was found as the only potential phoshporylation site, which might be a substrate for CDC2 kinase activity (suppl. Fig. 1A). This prediction was verified by an in vitro kinase assay. Herefore, CDC2 was co-immunoprecipitated from nocodazol blocked cells with either a specific Cyclin A or a Cyclin B antibody and incubated with bacterially expressed GST-Alienα together with γ-^{32}P-ATP. In these kinase assays, a band of about 60 kDa that

corresponds well to the size of GST-Alienα in an autoradiography screen was detected, which was not present in the negative control with GST alone (Fig 3C). Thus, this experiment revealed that Alienα was efficiently phosphorylated by CDC2 in vitro. Hereby, CDC2 was activated by Cyclin A as well as Cyclin B.
To assess possible consequences by Alienα phosphorylation, we generated mutants of Alienα in which serine 270 is replaced either by alanine or aspartic acid and expressed these Alienα mutants (Alienα Ser270Ala; Alienα Ser270Asp) in vitro. Afterwards, Alienα wt, Alienα Ser270Ala or Alienα Ser270Asp, respectively, were incubated with the bacterial expressed cullin-binding domain of APC2 (APC2 domain 2) that was previously immobilized onto nickel cellulose beads and a pulldown was performed. The bound Alienα forms were eluted from the beads, separated on a SDS gel and detected by western blot with a specific antibody. Thereby, we found only signals corresponding to both Alienα wt and the Alienα Ser270Ala mutant which seem to interact with APC2 domain 2, whereas the Alienα mutant mimicing phosphorylation (Alienα Ser270Asp) was not able to bind to APC2 (Fig. 3D). These results suggest, that the Alienα Ser270Asp mutant, which mimics a permanent phosphorylation, might be responsible for abrogation of the interaction with APC2.

Alienα increases genetic instability due to inhibition of mitotic cyclin degradation. Since the binding between Alienα and APC/C seems to depend on both the cell cycle and the phosphorylation state of Alienα it is likely that Alienα is able to alter cell cycle progression. First the DNA content of U2OS cells stably transfected with either Alienα, the alanine mutant, the aspartic acid mutant or, as negative control, mock transfected cells was measured by FACS analyses. Hereby we detected only slight alterations of the cell cycle (supplemental Fig. 1B). To further test the proliferation capacity of cells overexpressing Alienα a colony forming assay was performed. One thousand stably selected cells were plated per 6 well plates and cultured for two additional weeks. The cells transfected with Alienα wt, Alienα Ser270Ala or Alienα Ser270Asp, respectively, were fixed, stained and the colonies were counted. The cells transfected with Alienα wt showed a similar growth as the controls. Cells transfected with Alienα Ser270Ala increased colony number twice whereas the aspartic acid mutant (Alienα Ser270Asp) induced a significant decrease of colony numbers compared to Alienα wt. These data suggest that overexpression of the alanine mutant of Alienα triggered a generally faster cell cycle (Fig 4A).
Since Alienα overexpression alters the cell proliferation capacity it might lead to genetic instability. In order to proof this hypothesis stably transfected U2OS cells were cultured for about 4-5 weeks. Afterwards genetic alterations were measured with the high resolution array-CGH platform from Agilent. Genetic profiles of cells overexpressing Alienα wt or its mutants (Alienα Ser270Ala, Alienα Ser270Asp) were compared to the negative control, which was set as one. Gain as well as loss of genetic material was combined and two independent experiments were summarized (Fig. 4B). Consistent with our hypothesis the alanine mutant (Alienα Ser270Ala) which seems able to bind the APC/C permanently, aggregated the most genetic alterations whereas the aspartic acid mutant (Alienα Ser270Asp) exhibited nearly no differences compared to the mock transfected cells. If the cells accumulate genetic damages they might potentially possess a higher rate of apoptosis. Hence, we examined the caspase 3/7 activity of the cells in a further experiment. Hereby, the alanine mutant showed similar apoptosis rates as the mock transfected cells which were set as one (Fig. 4C). In contrast, Alienα and the aspartic acid mutant induced a decreased caspase activity. It seems that especially the Alienα form that mimics a phosphorylation (Alienα Ser270Asp) suppresses apoptosis by an as yet unknown mechanism.
Since the phosphorylation of Alienα seems to be necessary to disrupt its interaction with the APC/C in mitosis we expected that the phospho mutants are able to alter the degradation of the mitotic cyclins. To assess this hypothesis, transiently transfected cells were blocked in mitosis by nocodazole treatment. Cells were released from the block by addition of fresh medium and alterations in the concentration of Cyclin A as well as Cyclin B were monitored over 3 hours. Hereby, cells transfected with the Alienα Ser270Asp mutant showed a sharp decrease of the protein levels of the mitotic Cyclins A and B at the end of

mitosis, while the kinetic of degradation was far slower in cells transfected with the alanine mutant (Alienα Ser270Ala). In contrast, Alienα wt induced a faster degradation of Cyclin A, while the decay of Cyclin B1 was slowed down. Interestingly, overexpression of Alienα wt, Alienα Ser270Ala as well as Alienα Ser270Asp induced a reaccumulation of Cyclin A 3 hours after release from the nocodazole block. At this time point there was no band detectable in the mock transfected cells (Fig. 4D). Thus, it suggests that the mutation of Ser270 of Alienα regulates the stability of mitotic cyclins.

Alienα overexpression induced deregulation of the APC/C complex. These results suggest that Alienα is able to regulate the APC/C. To substantiate a potential role of Alienα in APC/C function we assessed the protein levels of three APC/C subunits, CDC27, APC1 as well as APC2, in cells stably transfected with either the wildtype Alienα or one of the Alienα phospho mutants, Alienα Ser270Ala or Alienα Ser270Asp, respectively. For that purpose, we analyzed the APC/C complex via a blue native PAGE and assessed the relevant subunits of the APC/C by specific antibodies in western blotting experiments (Fig. 5A). Hereby, we detected several bands corresponding to smaller subcomplexes of the APC/C containing the examined subunits of APC/C. The APC/C holocomplex could not be detected due to the use of detergents at lysis of the cells but we hypothesized that the concentration of the subcomplexes correlates to the amount of the holocomplex. Hereby we found that the constitutively phosphorylated form of Alienα, the Alienα Ser270Asp mutant, was able to downregulate APC1 as well as CDC27 containing APC/C subcomplexes whereas the concentration of APC2 at least in a smaller detected subcomplex was not affected (Fig. 5A). In case the alanine mutant (Alienα Ser270Ala) was stably transfected, complexes containing APC1, APC2 and CDC27 appeared in similar amounts compared to cells transfected with Alienα wt or a mock transfection using the empty vector (Fig. 5A). Afterwards, the issue was addressed how Alienα Ser270Asp reduces the stability of the APC/C. For this reason, the appearance of several monomeric subunits of APC/C were analyzed in U2OS cells stably transfected with expression vectors for Alienα wt, Alienα Ser270Ala or Alienα Ser270Asp, respectively. Cell extracts were separated in a denaturing SDS-PAGE and endogenous APC1, APC2 as well as CDC27 were analyzed by specific antibodies in immunoblot experiments (Fig. 5B). Both Alienα wt and Alienα Ser270Ala were not able to reduce the addressed proteins. In contrast, Alienα Ser270Asp induced a significant decrease of the signal corresponding to APC1. Additionally, Alienα Ser270Asp showed also an effect regarding protein stability of CDC27. The results suggest that Alienα Ser270Asp seems to be capable of both to influence the stability of APC/C as well as to downregulate at least two specific subunits of APC/C.

To analyze how Alienα reduces the cellular concentration of CDC27, first the effect on the transcription level of the *CDC27* gene expression was investigated. Hereby, an overexpression of Alienα as well as the phospho mutants did not induce a downregulation of *CDC27* mRNA levels (Fig. 5C). Next, cycloheximide chase experiments were performed to detect whether CDC27 is regulated by protein degradation. For this purpose U2OS cells transiently transfected with either Alienα wildtyp, Alienα Ser270Ala, Alienα Ser270Asp or, as negative control, the empty vector were used. After the protein synthesis was blocked by cycloheximide, the protein levels of CDC27 were analyzed for a period of 6 hours. Here we did not detect any effect on the degradation of CDC27 since the protein was stable for this period of time, independent on the treatment (Fig. 5D). Consistent with these results there were no differences in degradation of APC1 after transfection with Alienα Ser270Asp (suppl. Fig. 2A). These data are in contrast to the results received in stably transfected cells as shown in Fig. 5B. An explanation might be the existence of a kinetic effect in CDC27 degradation which is not trackable in short time analysis such as the cycloheximide chase experiment.

Apparently the cells were viable even after disruption of most of the APC/C subcomplexes. So there have to be mechanisms to keep the cell cycle stable. To get insight the expression of several cell cycle regulators in cells stably transfected with either Alienα or the empty vector, they were analyzed by a macroarray approach. Interestingly, the transcription of *CUL1, CDC20* and *BIRC5* was highly upregulated in cells

overexpressing Alienα (supplemental Fig. 2B). Cullin 1 is a part of the SCF complex and competes with the APC/C for ubiquitination of several targets [27]. This would explain the differences in stability of Cyclin A which is a target of both E3 ligases and showed opposite stability in cells either transiently or stably transfected with Alienα. However, as the APC/C is necessary for sister chromatid segregation this function seems to be mediated by the remaining small APC2 containing subcomplex and the CDC20 mediator, which is necessary for substrate specifity [28, 29]. Therefore, Alienα seems to be able to destabilize the APC/C complex as well as at least two of its subunits by an unknown mechanism. Additionally, Alienα is also able to keep a stable cell cycle even in the absence of this E3 ligase in mammalian cells. This may be due to the substitution of the APC/C function by the redundant SCF ubiquitination pathway and the stimulated upregulation of *CDC20*. Survivin which is encoded by the *BIRC5* gene is a known suppressor of apoptosis [30]. So the anti-apoptotic effect of Alienα might be mediated by survivin.

Discussion

The COP9 signalosome is a known regulator of the SCF E3 ligase system that controls the cell cycle progression by marking cyclins as well as their inhibitors for degradation [3]. In a very recent publication we showed that CSN2/Alienβ in context of the COP9 signalosome is seemingly able to control the anaphase promoting complex/cyclosome (APC/C). The physical binding of CSN2/Alienβ with the APC/C seems to be independent of its integration in the COP9 complex [14]. An explanation is that CSN2/Alienβ interacts with the cullin-like APC/C subunit APC2 directly through a site within its N-terminal 189 aa region [31, 32]. Consistent with these data, we show here that Alienα that represents the first 305 aa of CSN2/Alienβ also binds to the cullin domain of APC2 in vitro. Apparently both isoforms CSN2/Alienβ and Alienα compete for binding to the APC/C in vitro. In agreement with the competition, we found that several APC/C targets were reciprocally regulated by CSN2/Alienβ and Alienα. This effect induces an altered degradation for SnoN.

CSN2/Alienβ interacts with the APC/C in a cell cycle specific manner [14]. The present study suggests that Alienα binds also to the APC/C in interphase only when there is no interaction in mitosis. This would explain why the protein level of Cyclin B, which is degraded at the end of mitosis is not affected by Alienα [33, 34]. In this study we provided evidence that Alienα is phosphorylated in vitro by CDC2 and that an Alienα mutant containing an altered potential phosphorylation site (Ser270Asp) induced the disruption of the interaction between Alienα and APC2 in vitro. On the other hand, a mutation that abolishes the potential phosphorylation site did not affect the binding of Alienα to the APC/C. Therefore, the data indicate that the Alienα - APC/C interaction seems to be regulated during cell cycle by the phosphorylation status of Alienα.
Since the APC/C possesses a large number of interaction partners it seems likely that its regulation by Alienα is due to alterations in the complex composition. Apparently stable overexpression of Alienα Ser270Asp, which mimics a constitutively phosphorylation destroyed all APC/C subcomplexes containing APC1 and CDC27. This disruption was caused by the reduction of the APC1 and CDC27 protein levels, whereas the cellular concentration of APC2 was unaffected. Surprisingly, there were no changes in mRNA level of *CDC27* as well as no rapidly occurred protein degradation detectable in cells stably overexpressing Alienα. An explanation might be that here a kinetic effect in CDC27 degradation exists that is only detectable in a long time analysis. In agreement with this, all APC/C subunits apart from the two adapters Cdh1 and CDC20 are stable as reported [27]. One possibility is that CDC27 is regulated at the translational level or that Alienα changes the turnover in a very slight manner that ultimately leads to a downregulation over longer time periods. Dephosphorylation of Alienα is apparently necessary to stabilize the APC/C. The destabilization of APC/C triggered by the aspartic acid mutant of Alienα (Alienα Ser270Asp) seems incomplete as a subcomplex containing APC2 remains stable. This is in accordance to data by others that described also a stable subcomplex containing APC2 [35]. The APC2 subcomplex containing comprises a minimal ubiquitin ligase module of the APC/C that is still able to destruct both Cyclin B1 and

securin [29]. Furthermore, *CDC20* was upregulated at the transcriptional level after Alienα overexpression. Increase of this adaptor leads to a higher specificity for targets like Cyclin B and securin. Additionally, a higher expression of *CUL1* was detected. Cullin 1 is part of the SCF ubiquitination pathway and is able to mark several APC/C targets for degradation [29]. Coherent to these data we found that cells overexpressing the Alienα Ser270Asp mutant still triggered a delayed but appropriate cell cycle even after disruption of most of the APC/C subcomplexes. This might be due to an additional anti-apoptotic effect by Alienα as shown here because other cell lines display apoptotic behavior after depletion of APC1 [36]. The prevention of apoptosis might be mediated by the transcriptional upregulation of survivin. In addition, we hypothesize that there is still a small, not detectable amount of APC1 after overexpression of Alienα Ser270Asp that helps to keep the cells alive.

The APC/C is a known regulator of the degradation of mitotic cyclins [33]. In the present study we found that both Cyclin A as well as Cyclin B degradation is inhibited by the unphosphorylated Alienα (Alienα Ser270Ala). Thus it is conceivable that the alanine mutant of Alienα binds to the APC/C during the whole cell cycle and might be able to influence their destruction even in mitosis. Higher concentrations of mitotic cyclins in the G1 phase could bypass cell cycle arrests with only slight changes in the cell cycle phase distribution [15, 37]. Hereby, the influence of Alien on the cell cycle is even more complex. CSN2 exhibits a high complexity since it regulates the SCF complex as well as the APC/C [13, 38]. There is a tight crosstalk between these two E3 ligases and at the moment it is unclear how Alienα acts between these systems. Furthermore, a potential influence of Alienα on many cell cycle regulators by its interaction with E2F transcription factors was described recently [39, 40]. Furthermore, COP9 is also involved in the regulation of transcriptional activity of distinct factors [41]. Thus Alienα is able to govern both the transcription as well as the ubiquitin dependent degradation of several cell cycle regulators by redundant pathways. Consistent with these data we show here that cells expressing the Ser270Ala mutant of Alienα possessed a higher proliferation capacity and accumulated more genetic damages. On the other hand, the aspartic acid mutant form of Alienα induced less genetic alterations.

Taken together, our data suggests that CSN2/Alienβ as well as Alienα is able to govern both complex formation and target protein stability of the APC/C. On one hand, constitutive phosphorylation of Serin270 of Alienα is able to disrupt one key factor of the cell cycle - the APC/C complex - but still keeps an appropriate alternate cell cycle. On the other hand, if the Alienα/APC2 interaction is not splitted during mitosis by phosphorylation of Alienα Cyclin A and Cyclin B are stabilized. This leads to faster cell proliferation as well as promotes the appearance of genetic instability. Therefore, a single point mutation of Alienα switches between different possibilities to pass through cell cycle and might be important for tumor development and progression.

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Figure legends

Fig. 1. Alienα and CSN2/Alienβ compete for interaction with the APC/C: (A) APC2 cDNA was divided into three fragments fused to a His-tag and bacterially expressed. Protein extracts were incubated with nickel beads and in vitro translated Alienα was added. Following sequential washes the proteins were eluted by boiling with SDS sample buffer. As a negative control nickel beads incubated only with in vitro translated Alienα were used (lane 1). Alienα was detected by an immunoblot using a specific antibody against Alienα (PepAk2). Hereby, we found that APC2 domain 2, which contains the cullin domain, bound effectively to Alienα (lane 3) whereas no specific band was detected in the negative control (lane 1). The other two APC2 fragments showed only a weak interaction with Alienα (lane 2 and 4). (B) Bacterial expressed Alienα, CSN2/Alienβ or both was added to U2OS cell extract and CSN2/Alienβ was co-precipitated using a specific antibody against APC1. Thereafter, precipitated proteins were separated by SDS-PAGE and CSN2/Alienβ was detected by immunoblots using a specific antibody. In a control experiment IgG was used as unspecific antibody for immunoprecipitation. (C) APC1 was captured from U2OS cell lysate, which was supplemented with increasing amounts of in vitro translated Alienα. Co-precipitated CSN2 was detected by a specific antibody in an immunoblot. An unspecific band detected by the CSN2 antibody is labeled by an asterisk. A densitrometric analysis is shown.

Fig. 2. Alienα and CSN2/Alienβ overexpression induced invers protein level alterations of several APC/C targets: (A) Alienα or CSN2/Alienβ was stably overexpressed in U2OS by transfection with pcDNA3-Alienα or pcDNA3-CSN2/Alienβ, respectively. The empty vector was used as negative control. Several endogenous APC/C target proteins including Cyclin A, Cyclin B, CDC6 and SnoN were analyzed in the treated cells by immunoblotting using specific antibodies. Analyses of Alienα, CSN2/Alienβ and β-actin were used as expression or loading control, respectively. (B) The chart summarizes the results of three independent immunoblot experiments as described in (A). The concentrations of the samples were compared to β-actin and the protein amount in cells transfected with the empty vector were set as one. (C) U2OS cells which were transiently transfected with either pcDNA3-Alienα or the empty vector were treated with cycloheximide [50 µg/ml] 36 hours after initial transfection. Afterwards, cells were harvested after the indicated time points and analyzed by immunoblots using sepcific antibody for SnoN. The result of the densitometical analysis is summarized in the chart. (D) For analysis of the proliferation capacity, U2OS cells were stably transfected with Alienα, CSN2/Alienβ or the empty vector. 1000 cells were replated and grown for additional two weeks. Afterwards, the colonies were fixed, stained with Giemsa and colony numbers were counted. Three independent approaches were carried out. (E) Apoptosis rate of U2OS cells transiently transfected with pcDNA3-CSN2/Alienβ or the empty vector, respectively, was monitored with the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) in three independent approaches.

Fig. 3. The interaction between Alienα and APC1 occurred in a cell cycle dependent manner and was regulated due to phosphorylation of Alienα by cdc2: U2OS cells were synchronized in specific cell cycle phases and appropriate cell extracts were immediately used for CoIP experiments using a specific APC1 antibody or, as negative control, an unspecific antibody. A band corresponding to Alienα was detected in immunoblot using the specific Alienα antibody (lanes 1; 3-8), which was absent in the negative control (lane 2). As a control for equal protein loading corresponding β-actin levels were shown. Furthermore, the blot was analyzed densitometrically and the amount of Alienα bound to APC1 was plotted is depicted. (B) 36 hours

after transfection of HEK293 cells with pHA-linker (HA) or pHA-Alienα (HA-Alienα) respectively the medium was withdrawn and the cells were grown for another 12 hours in phosphate free medium. Following every dish was incubated with 600 mCi 32P-ortho-phosphate for 6 hours. The cells were lysed and HA-tagged proteins were immunoprecipitated from the supernatant using HA-hybridoma antibody. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by autoradiography. (C) For purification CDC2 was co-immunoprecipitated with a specific antibody either for Cyclin A or Cyclin B (kindly provided by C. Cales, IIB, Madrid, Spain) from the HeLa cell lysates. Following bacterially expressed Alienα was incubated with CDC2 in the presence of radioactive ^{32}P-ATP. After separation on an SDS gel the phosphorylated proteins were detected by an autoradiography using an X-ray film. * unspecific autoradiography signals (D) Bacterial expressed His-tagged APC2 domain 2 was bound to nickel beads and incubated with in vitro translated Alienα (Alienα wt, Alienα Ser270Ala or Alienα Ser270Asp, respectively). After sequential washes the proteins were separated on an SDS gel and immunoblotted with a specific antibody recognizing Alienα. *Upper panel* Alienα wt (lane 2) as well as the Alienα Ser270Ala mutant (lane 3) but not the Alienα Ser270Asp mutant (lane 4) was able to bind to the culline domain of APC2. *Below panel* A specific control immunoblot showed that comparable amounts of all three forms of Alienα were used in the pulldown assay.

Fig. 4. Regulation of the cell cycle and the genomic stability by Alienα is dependent on Alienα phosphorylation state: (A) U2OS cells were stable transfected with Alienα wt, Alienα Ser270Ala, or Alienα Ser270Asp, respectively and replated in a 6 well plate. Afterwards the cells were cultured for further two weeks under selection with G418. After fixing with methanol the cell colonies were stained with Giemsa and counted. In the chart three independent cell viability assays are summarized (upper panle). The picture shows a representative result of these experiments (below panel). (B) U2OS cells which were stably transfected with either pcDNA3-Alienα wt, pcDNA3-Alienα Ser270Ala, pcDNA3-Alienα Ser270Asp or the empty vector were growing for about a month. Afterwards, the number of genetic alterations was determined by an Array-CGH analysis using the Human Genome Microarray Kit 105A. The copy number of genes in the samples was compared to the cells transfected with the empty vector. In the chart genetic alterations including both loss and gain of genetic material of two independent experiments are summarized. (C) In U2OS cells stable transfected with Alienα wt, Alienα Ser270Ala, or Alienα Ser270Asp, respectively, were used to analyse the caspase3/7 activity. Samples were normalized to the cells transfected with the empty vector. (D) Wildtyp Alienα, Alienα Ser270Ala, or Alienα Ser270Asp, respectively was transiently overexpressed in U2OS cells. Afterwards, transfected cells were fixed in mitosis by treatment with nocodazole overnight. On the next day cells were harvested, washed with PBS and replated in fresh medium for the indicated time spans. After lysis the protein levels of Cyclin A and Cyclin B were monitored by immunoblot by specific antibodies.

Fig. 5. Phosphorylation of Alienα destabilized the APC/C: (A) U2OS cells were stably transfected with Alienα wt, Alienα Ser270Ala or Alienα Ser270Asp, respectively, lysed and protein lysate was separated under non-denaturing conditions by blue native PAGE experiments. The gels were assessed regarding the appearance of APC/C subcomplexes by immunoblot using a specific antibody against APC1 (upper panel), APC2 (middle panel) or CDC27 (below panel), respectively. Complexes containing APC1, APC2 or CDC27, respectively, were labeled by arrows. Actin immunoblot was used as loading control. (B) Alienα wt, Alienα Ser270Ala, Alienα Ser270Asp, or, as negative control, the empty vector were stably transfected in U2OS cells. Afterwards, the cells were lysed and the lysates were seperated on a denaturing SDS gel and APC/C subunits were analyzed by immunoblot experiments using specific antibodies. Both APC1 and CDC27 were detected with decreased signal intensity from cells transfected with Alienα Ser270Asp (lane 4) compare to cells stably transfected with Alienα or Alienα Ser270Ala

(lane 2; 3). Actin immunoblot was used as loading control. (C) U2OS cells stably transfected as in (B) were analyzed for their expression level of CDC27 mRNA by quantitative real-time PCR. The amount of *CDC27* mRNA was compared with the actin transcript level; the value of cells transfected with the empty vector was set as one. (D) U2OS cells were transiently transfected with Alienα wt, Alienα Ser270Ala, Alienα Ser270Asp, or, as negative control, the empty vector. The protein levels of CDC27 at the indicated time points after cycloheximide chase were analyzed by immunoblot using a specific CDC27 antibody.

Supplemental Fig. 1. (A) Potential phosphorylation sites of Alienα were predicted by an online database (http://csbl.bmb.uga.edu/~ffzhou/gps_web/predict.php). Hereby, Ser270 was found as the only potential site for phosphorylation by a cyclin dependent kinase. (B) The cell cycle distribution of U2OS cells transiently overexpressing either Alienα wt, Alienα Ser270Ala, Alienα Ser270Asp or, as negative control, the empty vector was analyzed with flourescence activated cell sorting (FACS). All RNA was degraded using RNase A and the DNA was stained with propidium iodide. None of the treatments possessed a significant impact on the cell cycle distribution. The graphs show one representative example of three independent experiments.

Supplemental Fig. 2. (A) Since CDC27 showed no changes in degradation after transient transfection with Alienα the cycloheximide chase was repeated with APC1. U2OS cells were transiently transfected as described above and the cells were harvested at the indicated time points after addition of cycloheximide. Thereafter APC1 protein levels were monitored by immunoblot. There was no difference in degradation between the negative control, Alienα wt and its phosphomutants. Like CDC27 APC1 was completely stable during the analyzed time span. (B) Changes of several regulators of cell cycle were monitored using the Oligo GEArray Human Cell Cycle microarray. Total RNA of cells stably transfected with Alienα or the empty vector was isolated and hybridized on the microarray. There were several changes in expression of cell cycle regulators detectable. *CDC20, CUL1* and *BIRC5* were upregulated after overexpression of Alienα.

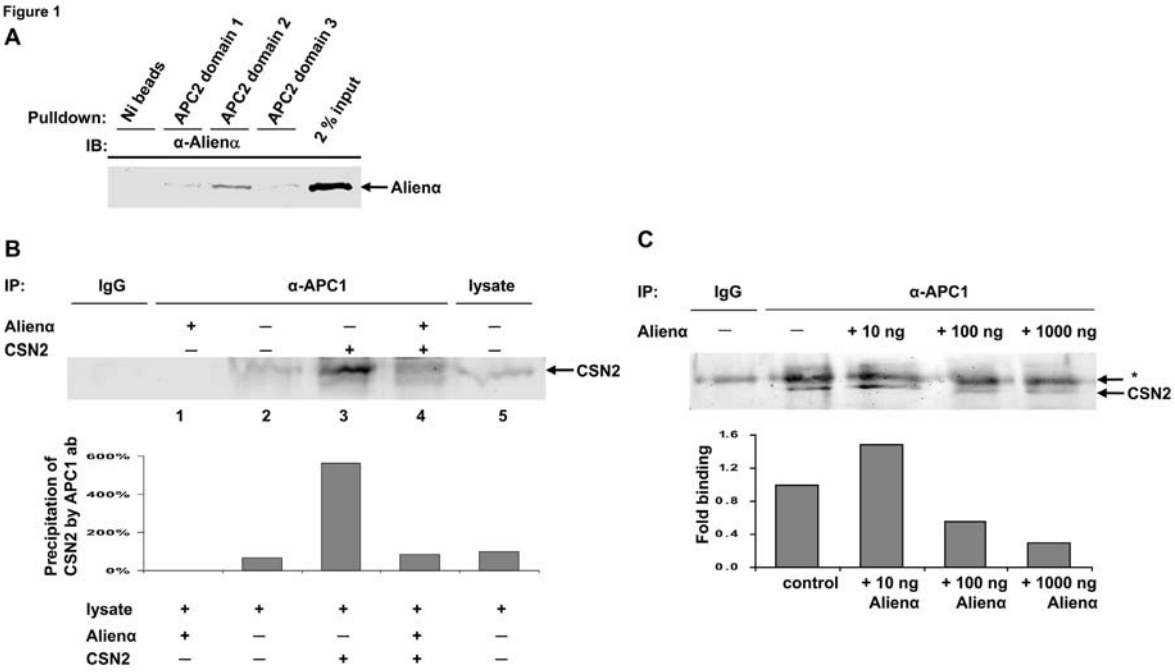


Figure 1
A
Pulldown:
Ni beads
APC2 domain 1
APC2 domain 2
APC2 domain 3
2 % input
IB:
α-Alienα
Alienα
B
IP:
IgG
α-APC1
lysate
Alienα
CSN2
CSN2
1
2
3
4
5
Precipitation of CSN2 by APC1 ab
600%
400%
200%
0%
lysate
Alienα
CSN2
C
IP:
IgG
α-APC1
Alienα
+ 10 ng
+ 100 ng
+ 1000 ng
*
CSN2
Fold binding
1.6
1.2
0.8
0.4
0.0
control
+ 10 ng Alienα
+ 100 ng Alienα
+ 1000 ng Alienα

Figure 2

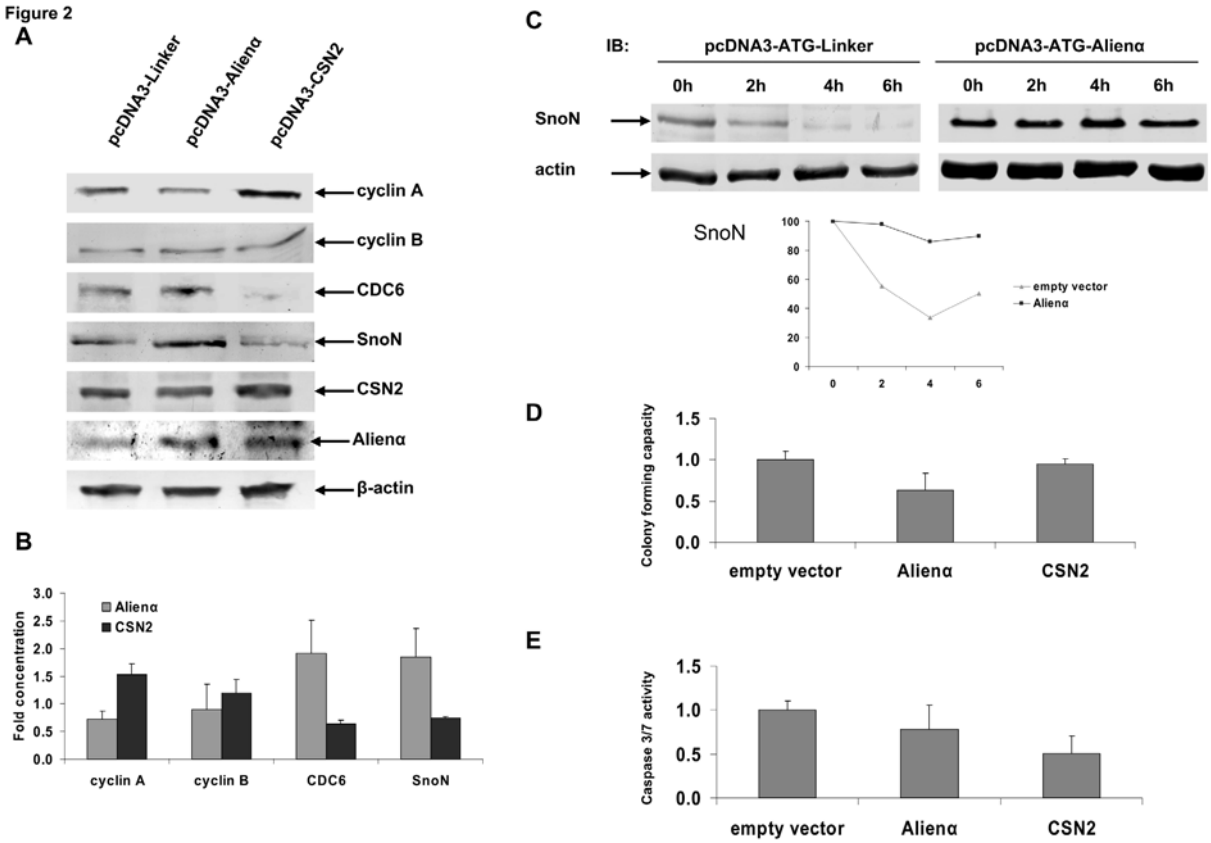

A
pcDNA3-Linker
pcDNA3-Alienα
pcDNA3-CSN2
cyclin A
cyclin B
CDC6
SnoN
CSN2
Alienα
β-actin
B
Alienα
CSN2
Fold concentration
3.0
2.5
2.0
1.5
1.0
0.5
0.0
cyclin A
cyclin B
CDC6
SnoN
C
IB:
pcDNA3-ATG-Linker
pcDNA3-ATG-Alienα
0h
2h
4h
6h
SnoN
actin
SnoN
100
80
60
40
20
0
0
2
4
6
empty vector
Alienα
D
Colony forming capacity
1.5
1.0
0.5
0.0
empty vector
Alienα
CSN2
E
Caspase 3/7 activity
1.5
1.0
0.5
0.0
empty vector
Alienα
CSN2

Figure 3

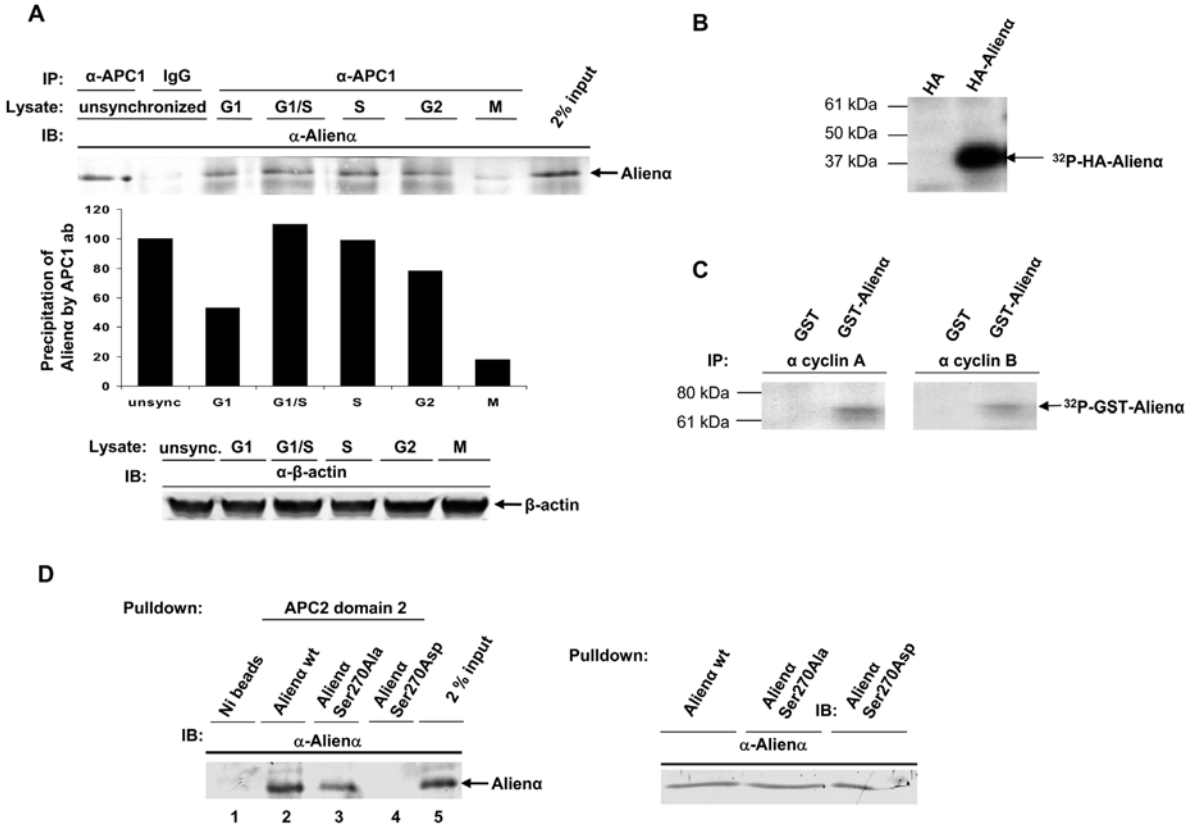

A
IP: α-APC1 IgG α-APC1
Lysate: unsynchronized G1 G1/S S G2 M 2% input
IB: α-Alienα
Alienα
Precipitation of Alienα by APC1 ab
120
100
80
60
40
20
0
unsync G1 G1/S S G2 M
Lysate: unsync. G1 G1/S S G2 M
IB: α-β-actin
β-actin
B
HA HA-Alienα
61 kDa
50 kDa
37 kDa
32P-HA-Alienα
C
GST GST-Alienα GST GST-Alienα
IP: α cyclin A α cyclin B
80 kDa
61 kDa
32P-GST-Alienα
D
Pulldown: APC2 domain 2
Ni beads
Alienα wt
Alienα Ser270Ala
Alienα Ser270Asp
2 % input
IB: α-Alienα
Alienα
1 2 3 4 5
Pulldown:
Alienα wt
Alienα Ser270Ala
IB:
Alienα Ser270Asp
α-Alienα

Figure 4

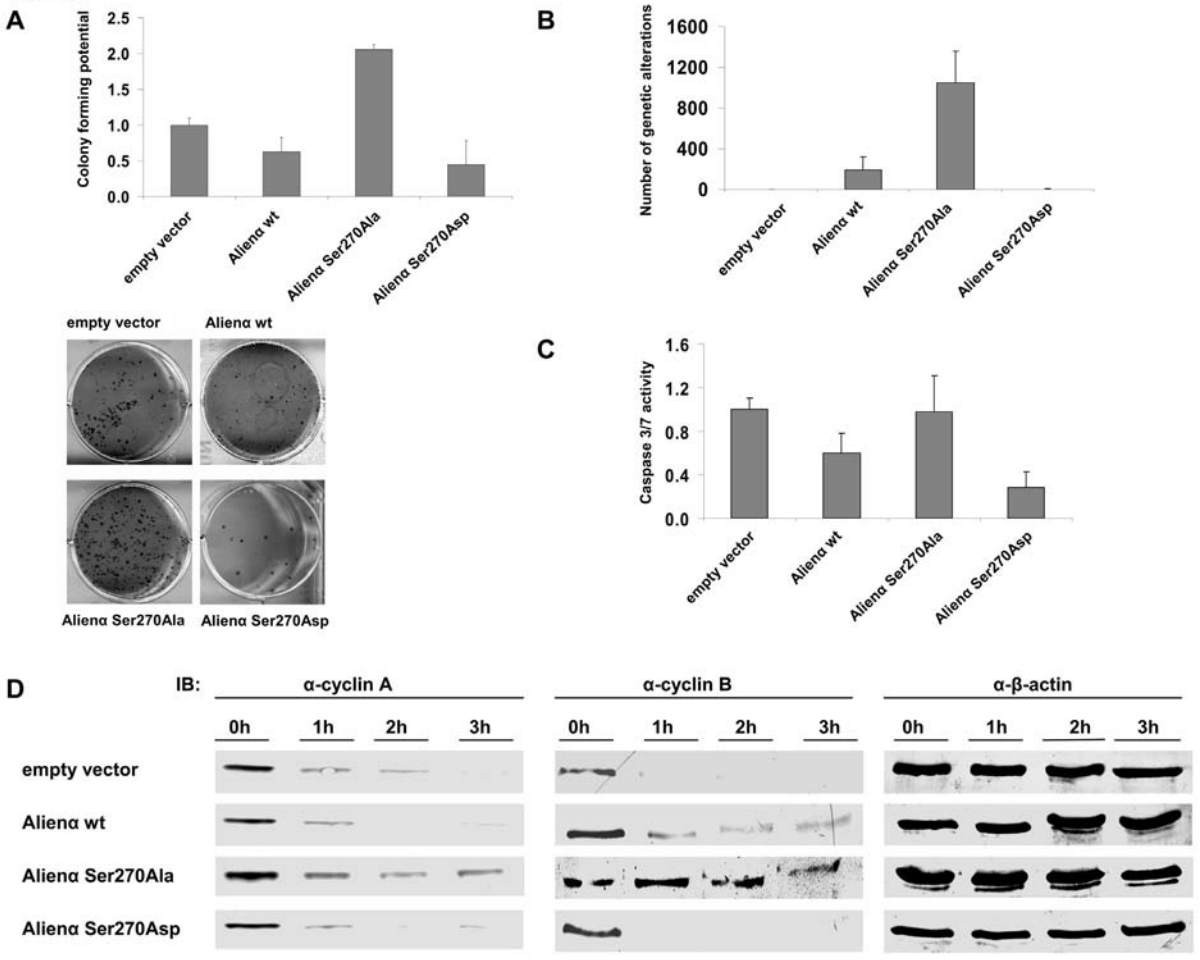
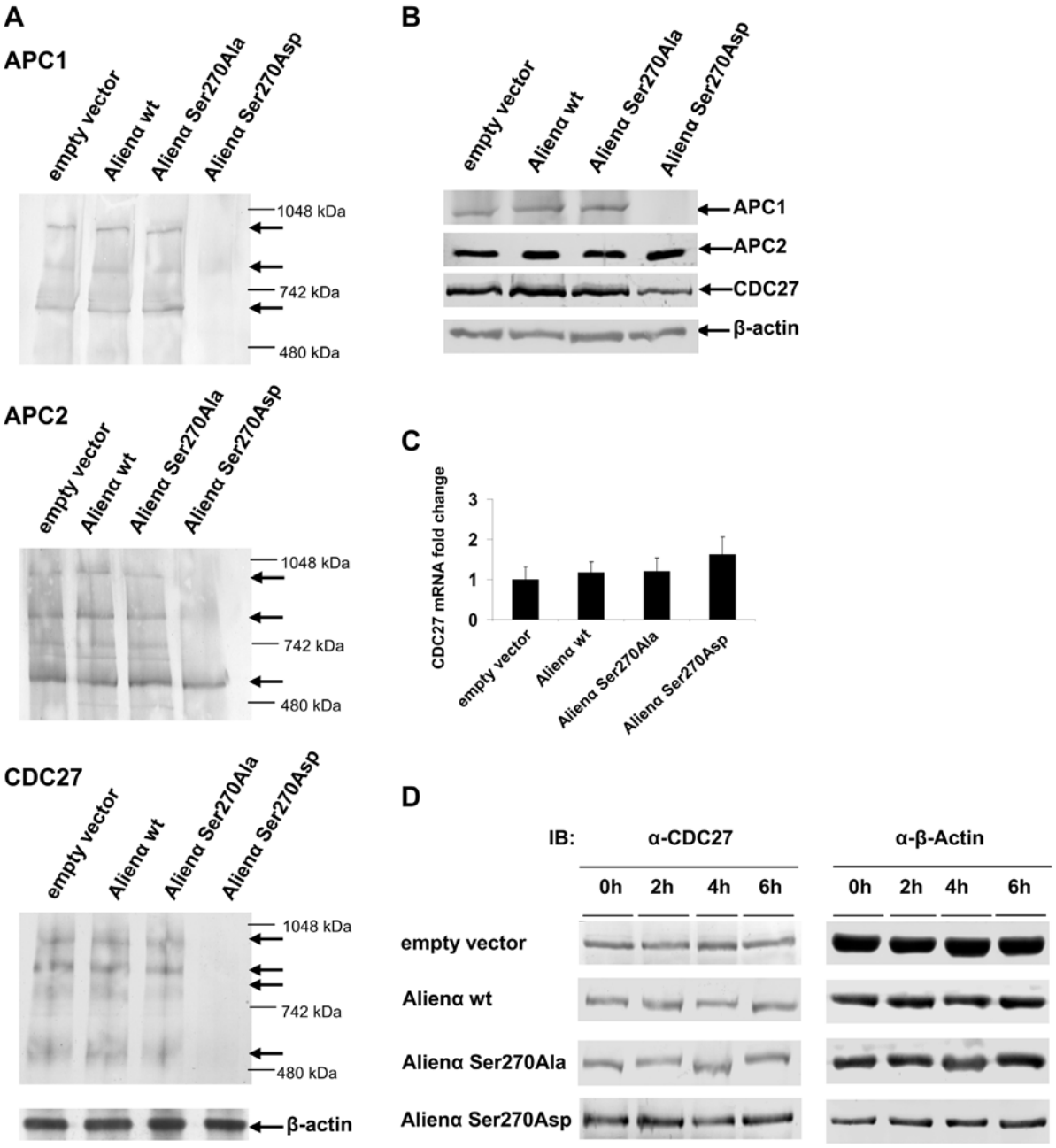

A
Colony forming potential
2.5
2.0
1.5
1.0
0.5
0.0
empty vector
Aliena wt
Aliena Ser270Ala
Aliena Ser270Asp
empty vector
Aliena wt
Aliena Ser270Ala
Aliena Ser270Asp
B
Number of genetic alterations
1600
1200
800
400
0
empty vector
Aliena wt
Aliena Ser270Ala
Aliena Ser270Asp
C
Caspase 3/7 activity
1.6
1.2
0.8
0.4
0.0
empty vector
Aliena wt
Aliena Ser270Ala
Aliena Ser270Asp
D
IB:
α-cyclin A
α-cyclin B
α-β-actin
0h
1h
2h
3h
empty vector
Aliena wt
Aliena Ser270Ala
Aliena Ser270Asp

Figure 5

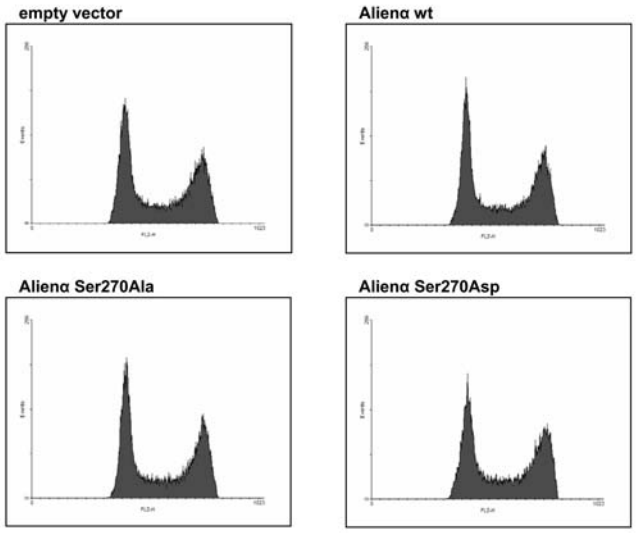

A
APC1
empty vector
Aliena wt
Aliena Ser270Ala
Aliena Ser270Asp
1048 kDa
742 kDa
480 kDa
APC2
CDC27
β-actin
B
APC1
APC2
CDC27
β-actin
C
CDC27 mRNA fold change
3
2
1
0
D
IB:
α-CDC27
α-β-Actin
0h
2h
4h
6h
empty vector
Aliena wt
Aliena Ser270Ala
Aliena Ser270Asp

Suppl. Fig. S1

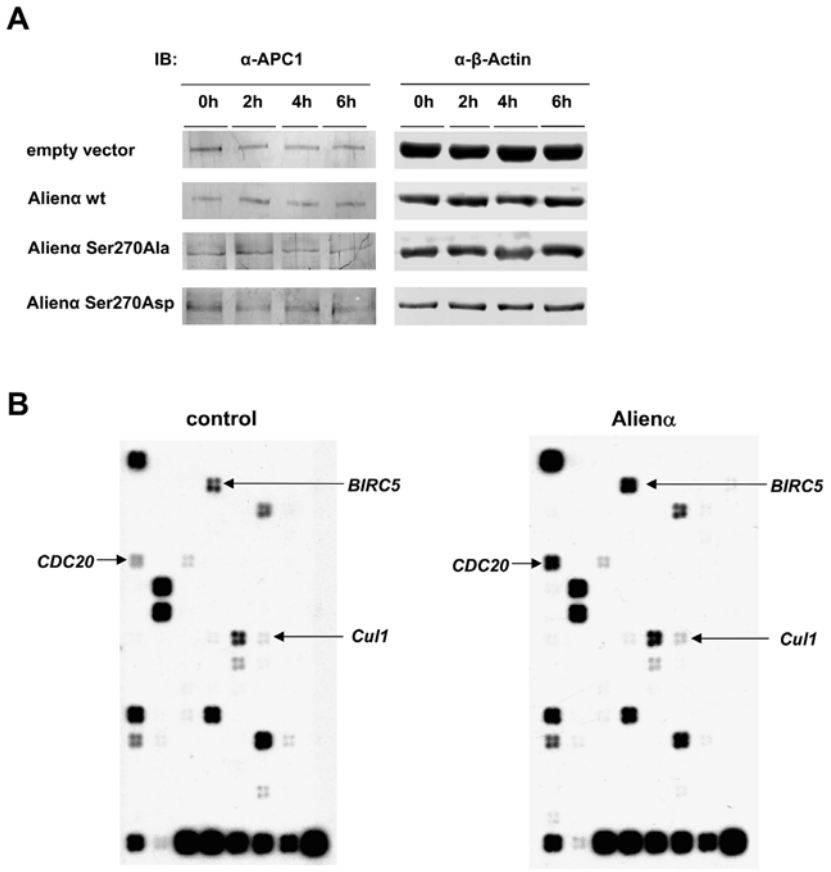
A

Position	Kinase	Peptide	GPS Score	Cutoff Score
270	P34CDC2	ESGSPRR	5.286	1.8

B



Suppl. Fig. S2


A
IB:
α-APC1
α-β-Actin
0h 2h 4h 6h
0h 2h 4h 6h
empty vector
Alienα wt
Alienα Ser270Ala
Alienα Ser270Asp
B
control
Alienα
BIRC5
CDC20
Cul1
BIRC5
CDC20
Cul1

3. Diskussion

Die Identifikation von neuen Protein-Protein-Interaktionen ist ein wichtiger Ansatz, um auf die biologische Funktion des untersuchten Proteins schließen zu können. Dabei wird davon ausgegangen, dass die physiologische Wirkung der meisten Proteine erst durch ihre Bindungspartner bestimmt wird [Coulombe *et al.*, 2004]. Viele Techniken für die Identifikation von neuen Interaktionspartnern, wie das Hefe-2-Hybridsystem, arbeiten mit hoch exprimierten Proteinen, die unter *in vitro* Bedingungen untersucht werden. Dies führt zu einer hohen Anzahl an falsch positiv oder falsch negativen Ergebnissen [Krause, 2006]. Darüber hinaus werden viele Interaktionen nur über die Wechselwirkung mehrerer Partner vermittelt [Gavin *et al.*, 2002]. Um diese Probleme auszuschliessen, wurde ein Interaktions-assay etabliert, der auf der Untersuchung von endogen exprimierten Proteinen beruht. Zur Absicherung der Ergebnisse werden dabei die mittels spezifischer Antikörper präzipitierten Proteine einmal anhand ihres Molekulargewichtes im Massenspektrometer und zusätzlich aufgrund ihres Peptid-Massen-Fingerabdrucks identifiziert. Anschließend werden diese neuen Bindungspartner mit einer Co-Immunpräzipitation bestätigt [Lehmann et al., 2005].

Um besser verstehen zu können, welche biologischen Prozesse durch Alien beeinflusst werden, wurde dieser Assay genutzt, um neue Interaktionspartner der beiden Alien-Isoformen zu identifizieren. Abschließend wurden einige der neuen Interaktionspartner mit Hilfe von Co-Immunpräzipitationen und anschließender Detektion mittels spezifischer Antikörper im Western Blot bestätigt. Dabei fällt aber auf, dass die Ergebnisse der Massenspektrometrie sich nicht vollständig mit den spezifischen Banden aus dem Gellauf decken. So konnten einige Signale des Massenspektrums der unverdauten Interaktionspartner nicht im Gel detektiert werden. Zusätzlich wiesen einige Banden im Gel keine Entsprechung im Massenspektrum auf. Dieser Umstand kann mit den methodischen Unterschieden in der Elution beziehungsweise der jeweiligen Detektion begründet werden. So werden nicht alle Proteine gleich gut im SELDI-Massenspektrometer ionisiert und dementsprechend ist die Detektion auch nicht quantitativ. Des Weiteren läuft ein Teil der Proteine im Gel nicht genau proportional zu seinem Molekulargewicht. Dadurch ist eine Zuordnung zu dem entsprechenden Massenspektrum erschwert. Hinzu kommt, dass ein Teil der Interaktionspartner eine ähnliche Größe wie die Antikörper-Fragmente aufweist und deshalb nicht als spezifisch erkannt werden kann. Trotz dieser methodischen Beschränkungen konnten mit der beschriebenen Vorgehensweise viele neue Bindungspartner von Alien identifiziert werden.

3.1 Identifizierung neuer Interaktionspartner von Alien

Die beiden Alien-Isoformen, CSN2 und Alienα, wurden in den letzten Jahren als Regulatoren von vielen verschiedenen Prozessen in der Zelle beschrieben. So wurde Alienα als Corepressor nukleärer Hormonrezeptoren wie dem Thyroidhormon-Rezeptor oder dem Vitamin-D3-Rezeptor charakterisiert [Polly *et al.*, 2000]. Corepressoren können die Transkription aktiv inhibieren ohne DNA direkt zu binden. Die Rekrutierung zu den spezifischen Zielgenen erfolgt mittels Transkriptionsfaktoren, die an den entsprechenden Promotoren lokalisiert sind. Viele Corepressoren können noch zusätzliche Faktoren wie Histondeacetylassen und Histonmethyltransferasen rekrutieren, die die Stilllegung der Genaktivität zusätzlich fördern [Burke & Baniahmad, 2000]. So konnte in Vorarbeiten eine durch Alienα induzierte Repression der transkriptionellen Aktivität von E2F-1 gezeigt werden [Escher *et al.*, 2007; Tenbaum *et al.*, 2007]. In Übereinstimmung mit seiner Bindung an den Thyroidhormon-Rezeptor konnte in der vorliegenden Arbeit eine Interaktion von Alienα mit dem Thyroidhormon-Rezeptor interagierenden Protein 11 (TRIP11) nachgewiesen werden [Kob *et al.*, 2007]. Dieses Protein ist ein Coaktivator des Thyroidhormon-Rezeptors und wirkt somit entgegengesetzt zu Alienα. Dabei interagiert Alienα mit dem Rezeptor in Abwesenheit des T3-Hormons, während TRIP11 nur im Zusammenspiel mit dem Hormon binden kann [Dressel *et al.*, 1999]. Aus diesem Grund bindet Alienα TRIP11 entweder unabhängig von dem Thyroidhormon-Rezeptor oder die Interaktion wird über ein Adaptor-Protein vermittelt. Hierbei könnte das Rb-Protein eine entscheidende Rolle spielen, da es beide Proteine bindet und als Repressor des Thyroidhormon beschrieben werden konnte [Chang *et al.*, 1997; Escher *et al.*, 2007].

Aufgrund der hohen Sequenzhomologie der beiden Alien-Isoformen erkennt der verwendete Antikörper sowohl CSN2 als auch Alienα. Bei der Bestätigung der in diesem Kapitel beschriebenen neuen Interaktionspartner wurde außer für TRIP11 nicht explizit nachgewiesen, welche Isoform gebunden war. Damit kann nicht bestimmt werden, welche der im Folgenden beschriebenen Interaktionspartner nur an CSN2 oder nur an Alienα oder an beide binden.

Des Weiteren wurden die beiden Untereinheiten VDRIP und CRSP3 des Vitamin-D-Rezeptor-interagierenden-Protein-Komplexes (VDRIP-Komplex) als Bindungspartner von Alien identifiziert. Dieser Komplex kann an mehrere nukleäre Hormonrezeptoren binden und deren Transkriptionsaktivierung nach Bindung des Liganden verstärken [Rachez *et al.*, 1999]. Auch in diesem Fall wechselwirkt Alien mit Aktivatoren der nuklären Hormonrezeptoren. Anscheinend existiert keine vollständig klare Trennung der Bindung von Coaktivatoren und

Corepressoren an die nukleären Hormonrezeptoren, da beide Faktoren auch miteinander interagieren. Wie aber die Wechselwirkung dieser beiden Gegenspieler erfolgt und reguliert wird, konnte bisher nicht geklärt werden.

Darüber hinaus wurde in dieser Arbeit gezeigt, dass Alien an die zwei Untereinheiten ERCC3 und p44 des TFIIH Subkomplexes der RNA-Polymerase II binden kann. Die RNA-Polymerase II wiederum wird durch den VDRIP-Komplex beziehungsweise auch durch den aktiven Thyroidhormon-Rezeptor an aktive Promotoren rekrutiert und ermöglicht dann die Transkription des entsprechenden Gens [Ptashne & Gann, 1997; Ryu *et al.*, 1999; Liu *et al.*, 2005]. Damit kann vermutet werden, dass Alien diese Genaktivierung beeinflussen kann. Neben seiner Funktion als genereller Transkriptionsfaktor ist der TFIIH-Komplex auch in die Reparatur von DNA-Schäden involviert [Reardon & Sancar, 2004]. Dabei wird das COP9-Signalosom, und damit CSN2, zusammen mit TFIIH zum DNA-Schaden rekrutiert und ist für die Reparatur essentiell notwendig [Groisman *et al.*, 2003; Fousteri *et al.*, 2006].

Die hier gezeigte Interaktion mit B23/Nucleophosmin ist nur schwer funktionell zu deuten, da dieses Protein in sehr viele verschiedene Signalwege integriert ist [Okuda, 2002]. So wurde unter anderem eine Beteiligung von B23 an der Zellzyklusregulation, der Apoptose über p53-Aktivierung und dem Rb-Signalweg nachgewiesen [Takemura *et al.*, 1999; Falini *et al.*, 2005; Li *et al.*, 2006; Lin *et al.*, 2006]. Es konnte nachgewiesen werden, dass Alienα ebenfalls mit mehreren Proteinen, die am E2F/Rb Signalweg beteiligt sind, funktionell wechselwirkt [Escher *et al.*, 2007; Tenbaum *et al.*, 2007]. Damit wäre eine gemeinsame physiologische Funktion von Alienα und B23 in dem E2F/Rb Signalweg möglich.

Des Weiteren konnte in dieser Arbeit erstmals eine Interaktion von Alienα mit p33ING1 und p33ING2 beschrieben werden [Fegers *et al.*, 2007]. Darüber hinaus wurde gezeigt, dass die Überexpression von p33ING1 und p33ING2 eine verstärkte transkriptionelle Repression durch Alienα ermöglicht. Sowohl p33ING1 als auch p33ING2 wurden als Transkriptionsrepressoren beschrieben und können Zellen in die Seneszenz führen [Goeman *et al.*, 2005]. In Übereinstimmung damit wurde auch Alien als positiver Regulator der Seneszenz charakterisiert [Leal *et al.*, 2008].

Damit konnte der Assay zur Detektion von Interaktionspartnern unter Verwendung endogen exprimierter Proteine erfolgreich für die Suche nach neuen Bindungspartnern von Alien verwendet werden. Die Ergebnisse bestätigen die bisher beschriebenen physiologischen Funktionen Aliens als Transkriptionsregulator und erweitern sie um weitere Signaltransduktionswege.

3.2 Regulation des Anaphase promoting complex / Cyclosome (APC/C) durch CSN2

In einigen Publikationen wurde der Einfluss des COP9-Signalosoms auf das Ubiquitin / Proteasom-System untersucht. So konnte eine starke Homologie zwischen dem COP9-Signalosom und dem Deckel des 19S Proteasoms nachgewiesen werden [Li & Deng, 2003; Schwechheimer, 2004]. Anscheinend konkurrieren die beiden Komplexe um die Bindung an das 26S Proteasom und modifizieren so dessen Substratspezifität *in vitro* [Huang *et al.*, 2005]. Die bisher vorliegenden Daten basieren aber nicht auf endogen exprimierten Proteinen und könnten somit Artefakte darstellen. Mit Hilfe der beschriebenen Interaktionsstudien konnten vier Untereinheiten der Basis des 19S Proteasoms (SUG1, TBP1, S4 und 26S Proteasom-Untereinheit S5B) und eine Untereinheit des 20S Proteasoms (20S Proteasom-Untereinheit α6) als Bindungspartner von CSN2 präzipitiert werden [Kob *et al.*, 2009]. Im Anschluss wurden diese Interaktionen - außer der 26S Proteasom-Untereinheit S5B - mit Hilfe von Co-Immunpräzipitationen und dem spezifischen Nachweis im Western Blot bestätigt. Damit konnte zum ersten Mal gezeigt werden, dass endogen exprimiertes CSN2 *in vivo* sowohl mit der Basis des 19S Proteasoms als auch mit dem 20S Proteasom interagiert.

In einem weiteren Interaktionsassay mit einem CSN2-spezifischen Antikörper wurden die Untereinheiten APC1, APC4 und APC6 des Anaphase promoting complex / Cyclosome (APC/C) als potentielle Bindungspartner von CSN2 identifiziert. Der APC/C ist neben dem SCF-Komplex hauptsächlich an der Markierung von Zellzyklusregulatoren mit Ubiquitin zur Degradation im 26S Proteasom beteiligt. Das Substratspektrum reicht dabei von Cyclinen (Cyclin A, Cyclin B), Untereinheiten verschiedener Signaltransduktionswege (SnoN, Ets2), DNA-Replikation-Regulatoren (Geminin, Cdc6) bis zu Proteinen, die an der Spindelformation beteiligt sind (Securin, XKid) [Harper *et al.*, 2002; Li & Zhang, 2009].

Die physikalische Interaktion zwischen CSN2, dem Proteasom und dem APC/C wurde näher charakterisiert. Der beschriebene Interaktionstest kann nicht zwischen direkten und über zusätzliche Proteine vermittelten Interaktionen unterscheiden. Damit die biologische Funktion von CSN2 weiter beschrieben werden kann, wurde deshalb die Bindung des COP9-Komplexes an das Proteasom und den APC/C untersucht. Dafür wurde der Proteasomeninhibitor N-Ethylmaleimid (NEM) eingesetzt, der zu einer Auflösung des COP9-Komplexes in seine Untereinheiten führt [Hetfeld *et al.*, 2005]. Damit kann nachgewiesen werden, ob die Bindung von CSN2 an das Proteasom beziehungsweise den APC/C direkt oder nur als Bestandteil des COP9-Komplexes erfolgt. Stellvertretend wurden die Untereinheit APC1 für den APC/C-Komplex und SUG1, das auch als TRIP1 bezeichnet wird, für die Basis

des 19S Proteasoms untersucht. Hierbei stellte sich heraus, dass die Interaktion von CSN2 mit SUG1, die in unbehandelten U2OS-Lysat nachgewiesen werden konnte, durch die Inkubation des Lysates mit NEM aufgehoben wurde. Dieses Ergebnis konnte sowohl durch eine Präzipitation mit spezifischen Antikörpern gegen SUG1 als auch gegen CSN2 mit anschließender Detektion des jeweils anderen Interaktionspartners im Western Blot bestätigt werden. Damit konnte gezeigt werden, dass der COP9-Komplex allerdings nicht dessen CSN2-Untereinheit alleine an die Basis des 19S Proteasoms binden kann. Im Gegensatz dazu war ein spezifischer APC1-Antikörper in der Lage, sowohl ohne als auch mit Zusatz von NEM sowohl CSN2 als auch SUG1 zu präzipitieren. Damit ist es wahrscheinlich, dass diese Bindung jeweils unabhängig von einem intakten COP9-Signalosom ist.

Hierfür bieten sich zwei Erklärungsmöglichkeiten an. Erstens könnte der APC/C sowohl von dem COP9-Komplex als auch von dem Deckel-Subkomplex des 19S Proteasoms rekrutiert werden und dementsprechend der Abbau der entsprechenden Targets beeinflusst werden. Alternativ könnte der APC/C unabhängig von den beiden Komplexen an das Proteasom binden und seine Funktion spezifisch erfüllen. In diesem Fall wären der Deckel-Subkomplex und das COP9-Signalosom nicht essentiell nötig und würden nur bei Bedarf die Substratubiquitinierung und die anschließende Degradation modifizieren [Lee *et al.*, 2005]. In beiden Fällen bilden sich hochmolekulare Proteinkomplex-Konglomerate, die eine schnelle Ubiquitin-Markierung und Degradation von Substraten ohne Entweichen von Intermediaten garantieren können [Peng *et al.*, 2003]. Zusätzlich ist es möglich, vielfältigste zelluläre Signale zu integrieren und somit eine bedarfsgerechte Modulation des Ubiquitin / Proteasom-Weges zu erreichen.

Die Beeinflussung der Substratumsetzung durch CSN2 wurde im Folgenden für die APC/C-Targets SnoN, Cyclin A, Cyclin B und CDC6 untersucht. Dazu wurden als erstes die zellulären Konzentrationen dieser Proteine nach Überexpression von CSN2 ermittelt. Die Hochregulation von CSN2 führt zur *de novo* Assemblierung des COP9-Komplexes [Huang *et al.*, 2005]. Damit sollte der Einfluss des Signalosoms auf den APC/C untersucht werden. SnoN und CDC6 lagen in niedrigeren Konzentrationen vor, während Cyclin A ein höheres Proteinniveau aufwies. Dagegen war keine Änderung für Cyclin B zu detektieren. Eine Reduktion des aktiven COP9-Komplexes durch knockdown der Untereinheit CSN5 mittels spezifischer CSN5 siRNA führte zu einer gegenteiligen Beeinflussung. Damit konnte gezeigt werden, dass CSN2 im Zusammenspiel mit dem COP9-Signalosom die Stabilität von APC/C Targets reguliert. Es stellte sich hierbei heraus, dass der COP9-Komplex den APC/C nicht generell inhibiert oder aktiviert, sondern eine Substratspezifität aufweist. So wurden die

Konzentrationen der untersuchten APC/C-Substrate nicht in die gleiche Richtung geändert, sondern der Einfluss war abhängig von dem entsprechenden Target. Um auszuschließen, dass der Effekt auf einer transkriptionellen Regulation beruht, wurde ein Cycloheximide-Chase-Experiment durchgeführt. Dabei wurde die Translation durch Cycloheximid geblockt und die Abnahme der Proteinkonzentrationen durch Degradation konnte über die Zeit mittels Western Blot sichtbar gemacht werden. Mittels dieses Ansatzes wurde der Einfluss von CSN2 auf den Abbau der untersuchten APC/C-Targets bestätigt. Es muss aber davon ausgegangen werden, dass die beobachteten Resultate möglicherweise durch das Zusammenspiel des APC/C mit dem SCF-Komplex herrühren, da sowohl Cyclin A als auch CDC6 Substrate beider E3-Ligasen sind [Nakayama & Nakayama, 2006].

In dieser Arbeit konnte gezeigt werden, dass CSN2 nicht mit dem APC/C während der Mitose interagiert. Dafür wurden U2OS-Zellen in den verschiedenen Zellzyklusstadien arretiert und anschließend eine Co-Immunpräzipitation mit einem spezifischen CSN2-Antikörper durchgeführt. Cyclin B wird am Ende der Mitose durch den APC/C ubiquitiniert und daraufhin abgebaut [Li *et al.*, 2007]. Da die Bindung von CSN2 an den APC/C in dieser Zellzyklusphase aufgehoben ist, könnte dies erklären, warum keine Regulation der Konzentration von Cyclin B durch den COP9-Komplex detektiert werden konnte.

Änderungen in der Konzentration der Cycline aber auch von CDC6 führen zu Fehlern in der Zellzyklusprogression und können somit zu genetischer Instabilität führen. Die Deregulation des APC/C kann hauptsächlich auf zwei Wegen zu diesen genetischen Veränderungen beitragen.

Einerseits ist dieser Komplex für den Spindel-Kontrollpunkt verantwortlich, der solange ein Voranschreiten von der Meta- zur Anaphase verzögert, bis sich alle Chromosomen an die mitotische Spindel angeheftet haben. Fehler in diesem Kontrollpunkt führen zu einer ungleichmäßigen Verteilung der Schwesterchromatiden in den Tochterzellen und somit zum Zugewinn oder Verlust ganzer Chromosomen [Wäsch & Engelbert, 2005]. Aufgrund der fehlenden Interaktion von CSN2 und dem APC/C in der Mitose und der unveränderten Degradationkinetik von Cyclin B waren Schäden durch diesen Mechanismus eher unwahrscheinlich.

Auf der anderen Seite kann eine, durch die Deregulation des APC/C hervorgerufene, erhöhte Cyclin-Aktivität in der G1-Phase und eine Veränderung der CDC6-Konzentration zu Fehlern in der Replikation führen. Hierbei akkumulieren DNA-Schäden in der S-Phase und es kann zu einer Re-Replikation kommen [Vaziri *et al.*, 2003]. In Übereinstimmung damit konnten in der vorliegenden Arbeit sowohl Verluste als auch Vervielfältigungen von

Chromosomenabschnitten nach einer stabilen Überexpression von CSN2 detektiert werden. Darüber hinaus wurde aber auch eine Beteiligung des COP9-Komplexes an der DNA-Schadensreparatur und der Replikation auf anderen Wegen als über den APC/C beschrieben, die unsere Ergebnisse ebenfalls erklären könnten [Groisman *et al.*, 2003; Richardson & Zundel, 2005]. Aufgrund der Komplexität der biochemischen Wege, die das COP9-Signalosom moduliert, kann hier nicht abschließend geklärt werden, welcher Anteil der DNA-Schäden durch eine Fehlregulation des APC/C hervorgerufen wird. Da CDC6 einen der entscheidenden Faktoren für eine korrekte Replikation darstellt, ist dessen Beitrag an genomischen Instabilitäten, die durch die Deregulation des COP9-Komplexes hervorgerufenen werden, wahrscheinlich sehr hoch.

3.3 Regulation des Anaphase promoting complex / Cyclosome durch Alienα

Alienα entsteht wahrscheinlich durch posttranskriptionelles Editing und repräsentiert die N-terminalen 305 Aminosäuren von CSN2 [Dressel *et al.*, 1999; Tenbaum *et al.*, 2003]. Damit fehlt Alienα die C-terminale PCI-Domäne, die für die Integration von CSN2 in den COP9-Komplex benötigt wird [Fu *et al.*, 2001; Huang *et al.*, 2005]. Die Bindungsstelle für Culline ist aber sowohl in Alienα als auch in CSN2 enthalten [Lyapina *et al.*, 2001; Yang *et al.*, 2002]. Eine direkte physikalische Interaktion des zur Cullin-Familie gehörenden APC2 an Alienα konnte in dieser Arbeit durch einen *in vitro* Interaktionsassay nachgewiesen werden. Aufgrund seines hohen Molekulargewichtes ist das vollständige APC2-Protein nur sehr schwer in Bakterien zu exprimieren. Daher wurde es in drei Domänen geteilt, von denen nur die Cullin-Domäne des APC2 an Alienα binden konnte. Angesichts dieser Ergebnisse war zu vermuten, dass Alienα und CSN2 um die Bindung an APC2 konkurrieren. Diese Kompetetion wurde in der vorliegenden Arbeit anhand eines *in vitro* Assays bestätigt.

Da anscheinend der gesamte COP9-Komplex, und nicht CSN2 alleine, den APC/C reguliert und Alienα nicht im Signalosom integriert ist, wurde untersucht, ob die Kompetition die Regulation des APC/C durch das COP9-Signalosom beeinflusst [Kob *et al.*, 2009; Kob et al., Manuskript zur Publikation eingereicht – siehe Kapitel 2.4]. Die Änderungen der Proteinkonzentrationen von Cyclin A, CDC6 und SnoN durch stabile Überexpression von Alienα entsprachen der Herunterregulation von CSN5 durch siRNA. Für SnoN wurde das höhere zelluläre Proteinniveau direkt mit einer langsameren Degradationskinetik nach der Überexpression von Alienα in Verbindung gebracht. Auf der anderen Seite führte die Überexpression von CSN2 zu dem gegenteiligen Ergebnis. Die Überexpression von CSN2

führt zu einer *de novo* Assemblierung des COP9-Komplexes, während der Verlust von CSN5 mit dem Verlust der meisten biochemischen Aktivitäten des Signalosoms, aber nicht mit dessen Spaltung, assoziiert wird [Huang *et al.*, 2005; Peth *et al.*, 2007]. Damit scheint Alienα durch seine Kompetetion mit CSN2 zu ähnlichen Effekten zu führen, wie eine direkte Inhibition des COP9-Komplexes durch Herunterregulation von CSN5.

Aus diesem Grund sollte die Zellzyklusabhängigkeit der Interaktion zwischen Alienα und dem APC/C untersucht werden. Ähnlich wie CSN2 zeigte Alienα eine Bindung an den APC/C von der G1- über die S- bis zur G2-Phase, während die Interaktion in der Mitose wesentlich schwächer war. Bei der Datenbanksuche nach Kinasen, die möglicherweise Alien während des Zellzykluses phosphorylieren, wurde CDC2 als möglicher Effektor ermittelt. Aufgrund der Konsensussequenz wurde das Serin an Position 270 als potentielle Phosphorylierungsstelle angegeben, die sowohl in CSN2 als auch Alienα vorhanden ist.

CDC2 zusammen mit Cyclin A und Cyclin B ist ein bekannter Regulator des G2/M-Übergangs, der Chromsomenkondensation und der Auflösung der Kernmembran [Lindqvist *et al.*, 2007; van Leuken *et al.*, 2008]. Diese cyclin-abhängige Kinase wird durch mehrere Faktoren reguliert und wird dadurch am Ende der G2-Phase aktiviert [Lindqvist *et al.*, 2009]. Diese Effekte werden zum großen Teil durch die Phosphorylierung des APC/C hervorgerufen. Durch diese Modifikation kann der Adaptor CDC20 an den APC/C binden und somit dessen Substratspektrum beeinflussen [Shteinberg *et al.*, 1999].

Durch einen *in vitro* Phosphorylierungsassay konnte die *in silico* Vorhersage, dass Alienα von CDC2 phosphoryliert wird, belegt werden [Kob *et al.*, 2009]. Um die Relevanz dieser Phosphorylierung für die Interaktion zwischen Alienα und APC2 nachzuweisen, wurde eine *in vitro* Bindungsstudie durchgeführt. Dabei zeigte sich, dass neben dem Alienα Wildtyp auch die Mutante (Alienα Ser270Ala), bei der das Serin durch ein Alanin ausgetauscht wurde, an die Cullin-Domäne des APC2 bindet. Dagegen war keine Interaktion mit der Mutante (Alienα Ser270Asp), die durch die Substitution des Serins durch eine Asparaginsäure eine konstitutive Phosphorylierung mimikrierte, nachweisbar. Damit liegt eine zeitliche Übereinstimmung zwischen dem Aktivitätsmaximum von CDC2 und der Repression der Interaktion zwischen APC2 und Alienα vor. Zusätzlich verliert Alienα die Bindungsfähigkeit an die Cullin-Domäne, wenn eine dauerhafte Phosphorylierung an der Consensussequenz des CDC2 in Alienα mimikriert wird. Damit ist diese Kinase anscheinend für die Regulation der Wechselwirkung von Alienα mit dem APC/C verantwortlich.

Cyclin B wird im Gegensatz zu Cyclin A, das bereits ab Ende der G2-Phase degradiert wird, erst im Verlauf der Mitose durch den APC/C mit Ubiquitin markiert und abgebaut [Yam *et al.*, 2000; Raff *et al.*, 2002; Acquaviva & Pines, 2006]. Da beide Alien-Isoformen während der Mitose nicht an den APC/C gebunden sind, ist das eventuell die Ursache, warum keine Änderungen im Proteinlevel von Cyclin B nach Überexpression von Alienα oder CSN2 gefunden wurden. Aus diesem Grund sollte untersucht werden, ob die Ser270Ala Mutante, die nicht durch CDC2 phosphoryliert werden sollte und deshalb während des gesamten Zellzykluses an den APC/C bindet, einen Einfluss auf die Degradationskinetik von Cyclin A und Cyclin B besitzt. Es konnte gezeigt werden, dass die Ser270Ala Mutante sowohl die Degradation von Cyclin A als auch Cyclin B am Ende der Mitose inhibiert. Der verzögerte Abbau von Cyclin B nach Transfektion mit dem Alienα Wildtyp könnte mit einer unvollständigen Phosphorylierung aufgrund seiner höheren zellulären Konzentration begründet werden. Interessant ist die frühere Reakkumulation von Cyclin A in allen drei Formen von Alienα, die untersucht wurden. Dieser Effekt wurde bereits auch nach Herunterregulation von CDH1 beschrieben [Bashir *et al.*, 2004]. Aus diesem Grund kann vermutet werden, dass selbst die Ser270Asp Mutante einen inhibitorischen Effekt gegenüber dem APC/C^{CDH1}-Komplex ausübt.

Der vollständige Abbau der Cycline am Ende der Mitose ist notwendig, um einen vorzeitigen Eintritt in die nächste S-Phase zu verhindern [Wäsch & Engelbert, 2005]. Die funktionellen Konsequenzen dieser erhöhten Cyclin-Aktivität reichen von einer generellen Beschleunigung des Zellzykluses bis zu verstärkter genomischer Instabilität [García-Higuera *et al.*, 2008]. Zusätzlich kann ein p27-abhängiger Zellzyklusblock in der G1-Phase durch mitotische Cycline übergangen werden [Sudo *et al.*, 2001]. Diese Daten stimmen mit der Verdopplung der Proliferationsfähigkeit nach Überexpression der Ser270Ala Mutante und der große Anzahl an genetischen Veränderungen überein. Dagegen führt die Ser270Asp Mutante zu einer Halbierung der Koloniebildungsfähigkeit und weist fast keine genetischen Unterschiede im Vergleich zu der mit dem Leer-Vektor transfizierten Kontrolle auf. Der Alienα Wildtyp nimmt sowohl in der Wachstumsfähigkeit als auch bei den genetischen Inbalancen eine Mittelstellung zwischen seinen beiden Mutanten ein. Interessanterweise zeigte die Ser270Ala Phosphorylierungs-Isoform trotz der Chromosomenschäden keine erhöhte Apoptoserate, während der Wildtyp und die Ser270Asp-Mutante sogar zu einer Reduktion der Caspase 3/7-Aktivität führten. Aus diesem Grund kann vermutet werden, dass Alienα die Apoptose auf einen bisher unbekannten Weg aktiv reprimiert.

Der APC/C wird durch die Bindung einer Vielzahl von Faktoren gesteuert [Thornton & Toczyski, 2006]. Damit ist zu vermuten, dass seine Regulation durch Alienα über die Änderung der Komplexstrukur erfolgt. Aus diesem Grund wurden mit Alienα oder einer der beiden Phosphomutanten stabil transfizierte Zellen auf nativen Gelen hinsichtlich der enthaltenen APC/C-Komplexe, die entweder APC1, APC2 oder CDC27 enthielten, analysiert. Dabei fiel auf, dass die Ser270Ala-Mutante zur Zerstörung der meisten APC/C Subkomplexe führte. Nur ein Subkomplex blieb stabil, der APC2 aber nicht APC1 oder CDC27 enthielt. Die Analyse der zellulären Konzentration dieser Proteine ergab, dass sowohl APC1 als auch CDC27 schwächer exprimiert vorlagen, während das APC2-Niveau unverändert war. Die Destabilisierung des APC/C-Komplexes erfolgte also durch die Herunterregulation der Proteinspiegel von mindestens zwei seiner Untereinheiten.

Der Mechanismus, der die Konzentration von APC1 und CDC27 vermindert, konnte bisher nicht identifiziert werden und muss Inhalt zukünftiger Forschungsarbeiten sein. Es war keine Änderung der *CDC27* Transkription mit Hilfe der quantitativen real time PCR nachzuweisen. Auch konnten in nachfolgenden Cycloheximide Chase Experimenten keine Änderungen der Degradationskinetiken von APC1 und CDC27 detektiert werden. In Übereinstimmung mit diesen Resultaten wurden alle Untereinheiten des APC/C außer den beiden Adaptorproteinen CDC20 und CDH1 als sehr stabil beschrieben [Vodermaier, 2004]. Aus diesem Grund könnten die verringerten Proteinspiegel damit erklärt werden, dass durch den geringen Umsatz von APC1 und CDC27 bereits sehr geringe Änderungen der Transkription oder des Abbaus, die mit den verwendeten Methoden nicht erfasst werden können, über längere Zeit zu den beobachteten Resultat führen. Zusammenfassend scheinen die zyklische Dephosphorylierung von Alienα und die damit verbundene Interaktion mit dem APC/C für dessen Stabilität notwendig zu sein.

Die Zellen, die die Ser270Asp Mutante überexprimierten, zeigten einen stabilen Zellzyklus. Damit wird die antiapoptotische Wirkung von Alienα belegt, da ohne die Überexpression der Ser270Asp Mutante die Depletion von APC1 auch in p53 negativen Zellen zur Apoptose führt [Teodoro *et al.*, 2004]. Dabei könnte der verbleibende, APC2 enthaltende Subkomplex wichtig für die Viabilität der Zellen sein. Ein stabiler APC2-Subkomplex wurde schon in anderen Arbeiten beschrieben [Thornton *et al.*, 2006]. Dieser Subkomplex enthält neben APC2 auch APC11 und bindet die entsprechenden E2-Ligasen. Damit stellt er die minimale Ubiquitinligase des APC/C dar und kann noch Securin und Cyclin B für den Abbau markieren [Tang *et al.*, 2001].

In Übereinstimmung damit führt die Überexpression von Alienα zu einer transkriptionellen Aktivierung des *CDC20*-, *BIRC5*- und des *Cul1*-Gens. CDC20 ist einer der Adaptorproteine des APC/C und ist für dessen Spezifität für mitotische Targetproteine wie Securin verantwortlich [Tang *et al.*, 2001]. Cul1 ist ein integraler Bestandteil des SCF-Komplexes, der ebenfalls Zellzyklusregulatoren wie zum Beispiel p27 für den Abbau im Proteasom markieren kann [Yang *et al.*, 2002; Bornstein *et al.*, 2006]. In Hefe konnte gezeigt werden, dass der Zellzyklus stabil bleibt, solange das Securin-Gen deletiert wird und das p27-Homolog Sic1 zum korrekten Zeitpunkt hochreguliert wird [Thornton & Toczyski, 2003]. Damit kann vermutet werden, dass Alien aufgrund seiner Wechselwirkung mit den SCF- und APC/C-Komplexen einen ähnlichen Einfluss ausübt. Survivin, das von dem *BIRC5*-Gen kodiert wird, ist ein bekannter Apoptoseinhibitor [Altieri, 2008]. Damit könnte die Viabilität und die verringerte Apoptose der Zellen trotz des Verlustes des APC/C erklärt werden.
Insgesamt konnte gezeigt werden, dass sowohl Alienα als auch CSN2 an den APC/C binden können und untereinander um diese Interaktion kompetieren. Dadurch wird die Degradation verschiedener Targetproteine beeinflusst. Diese Interaktion ist zellzyklusabhängig und kann durch die Phosphorylierung von Alienα durch CDC2 reguliert werden. Eine Deregulation dieser Phosphorylierung führt entweder zu einem beschleunigten Zellzyklus mit erhöhter genetischer Instabilität oder zur Zerstörung des APC/C-Komplexes mit geringerer Proliferationsfähigkeit der Zellen.

3.4 Alien als multifunktionaler Regulator

Rund 80% der Proteine sind in Komplexen organisiert, die durch wenige Proteine miteinander verbunden sind [Gavin & Superti-Furga, 2003]. So vernetzt CSN2 das COP9-Signalosom mit anderen Komplexen [Yang *et al.*, 2002]. Auch Alienα wurde als ein Faktor beschrieben, der Interaktionen zwischen verschiedenen Komplexen vermittelt [Papaioannou *et al.*, 2007]. Damit scheinen die beiden Alien-Isoformen wichtig für die Integration von Informationen aus verschiedenen Signaltransduktionswegen zu sein. Aufgrund dieser zentralen Stellung von Alien in der Kommunikation in der Zelle ist das Ausschalten des Alien-Gens bei Mäusen bereits prä-implatationsletal [Lykke-Andersen *et al.*, 2003]. Alien wurde in der vorliegenden Arbeit mit Regulationsmechanismen in Verbindung gebracht, die von der Transkription bis hin zur Degradation reichen. Damit konnte die wichtige Rolle von Alien als multifunktionaler Regulator bestätigt werden. Folglich könnte bereits eine leichte Deregulation der Alien-Aktivität zur Tumorenstehung und Progression beitragen.

4. Zusammenfassung

Die beiden Alien-Isoformen Alienα und CSN2 wurden in der Vergangenheit als Regulatoren verschiedenster zellulärer Prozesse identifiziert. Diese reichen von der Transkriptionskontrolle von Kern-Hormon-Rezeptoren, dem E2F/Rb-System, Beteiligung an verschiedenen Signaltransduktionswegen bis zur Regulation des Ubiquitin / Proteasom-Systems. Ziel dieser Arbeit war es mit Hilfe eines Interaktionsassays, der mit endogen exprimierten Proteinen arbeitet und immunologische mit massenspektrometrischen Methoden kombiniert, die Wechselwirkungen von Alien mit anderen Proteinen in der Zelle umfassender zu beschreiben. Anhand dieser Daten konnten sowohl bekannte Bindungspartner bestätigt als auch neue beschrieben werden.

Anhand dieses Interaktionsassays wurde die Kontrolle von nukleären Kernhormonrezeptoren durch Alien weiter untermauert und der Vitamin D abhängige VDRIP-Komplex konnte als Interaktionspartner beschrieben werden. Des Weiteren zeigte sich die Bindung von Alien an Proteine der allgemeinen Transkriptionsinitiation und DNA-Reparatur. Auch eine Wechselwirkung von Alienα mit den Tumorsuppressoren p33ING1 und p33ING2 wurde aufgezeigt. Damit wurde die Wichtigkeit von Alien für die transkriptionelle Kontrolle über eine Vielzahl von Wegen bestätigt.

Des Weiteren wurde der Einfluss von Alien auf das Ubiquitin / Proteasom-System untersucht. Dabei konnte zum ersten Mal die Interaktion der Alien-Isoformen mit dem APC/C beschrieben werden. Infolge der Deregulation des APC/C durch die Überexpression von Alienα beziehungsweise CSN2 wurden die zellulären Proteinkonzentrationen verschiedener Zellzyklusregulatoren, wie zum Beispiel Cyclin A, CDC6 und SnoN, verändert. Aufgrund der hohen Sequenzhomologie der beiden Alien-Isoformen konnte eine Kompetition zwischen Alienα und CSN2 für die Bindung an den APC/C gezeigt werden. In dieser Arbeit wurde nachgewiesen, dass wahrscheinlich die Phosphorylierung von Alienα durch CDC2 zur Inhibition der Interaktion mit dem APC/C in der Mitose führt. Auf der einen Seite konnte gezeigt werden, dass die Mimikrierung einer dauerhaften Phosphorylierung von Alienα die Bindung an den APC/C reprimiert. Dadurch wurden sowohl der APC/C Komplex als auch mehrere seiner Untereinheiten destabilisiert. Auf der anderen Seite führte die Deletion der Phosphorylierungsstelle zu einer dauerhaften Interaktion von Alienα und dem APC/C. Deshalb akkumulierten mitotische Cycline in der G1-Phase. Mit Hilfe von FACS-Messungen konnte nachgewiesen werden, dass dadurch der gesamte Zellzyklus schneller durchlaufen wurde. Darüber hinaus wurde mittels Array-CGH eine Anhäufung von genetischen Schäden

durch diese Deregulation des Zellzykluses gezeigt. Folglich kann eine Punktmutation in Alienα zum Umschalten zwischen einem langsamen, kontrollierten zu einem schnellen Zellzyklus mit vielen genetischen Schäden führen.

Zusammenfassend konnten in dieser Arbeit neue Interaktionspartner und physiologische Funktionen von Alien beschrieben werden. Dabei wurde sowohl seine Rolle als Transkriptionsfaktor, als auch bei der Regulation der Degradation von Proteinen bestätigt und um zusätzliche Wege erweitert. Damit konnte gezeigt werden, dass Alien in den Zellzyklus eingreift und für die genetische Stabilität und die Reduktion der Apoptose entscheidend ist. Aufgrund seiner Stellung als multifunktionaler Regulator zellulärer Prozesse könnte Alien somit für die Kontrolle der Tumorentstehung und Progression wichtig sein.

5. Synopsis

In the last years the Alien isoforms Alienα and CSN2 were described as regulators of diverse cellular functions. These range from the control of transcription by nuclear hormone receptors or the E2F/Rb system to the regulation of the ubiquitin / proteasome pathway. One aim of this work was to discover new interaction partners of Alien with a combination of immunological and mass spectrometric techniques. Solely endogen expressed proteins were analyzed to avoid false positive and negative results.

First it was shown that Alien binds to the VDRIP complex which mediates transcription. Besides, Alien was found to interact with several proteins of the general transcription initiation and DNA repair. Additionally, it could be shown that the two tumor suppressors p33ING1 and p33ING2 bind to Alienα and regulate its function. It was confirmed by this work that Alien is important for the transcriptional control by several pathways.

Furthermore the influence of Alien on the ubiqutin/proteasome system was examined. Hereby, the interaction of both Alien isoforms with the APC/C could be shown for the first time. By deregulating the APC/C through overexpression of Alienα as well as CSN2 the protein amount of several cell cycle regulators like Cyclin A, CDC6 and SnoN was altered. Due to the high homology of the two Alien isoforms a competition between Alienα and CSN2 for binding to the APC/C was detected. Furthermore, the phosphorylation of Alienα by CDC2 potentially inhibits the Alienα APC/C interaction during mitosis. On the one hand a mutation that mimics a constitutive phosphorylation of Alienα abolished its binding to the APC/C. Following, the APC/C complex as well as at least two of its subunits were destabilized. On the other hand deletion of the phosphorylation site leads to a permanent interaction of Alienα with the APC/C and higher concentrations of mitotic cyclins in the G1 phase. Hence, the whole cell cycle became faster. Furthermore, the cells accumulated more genetic damages as monitored by Array-CGH. Thereby the whole cell cycle is accelerated and the accumulation of genetic instabilities is induced. So a single point mutation of Alienα switches between a slow, regulated cell cycle or a fast cell cycle possessing many genetic alterations.

Taken together several new interaction partners and physiological functions of Alien could be described in the present work. These data confirm its importance for the ubiquitin / proteasome pathway as well as the influence on transcriptional regulation and extend our knowledge about the biological function of Alien. Deregulated Alien was shown to alter the cell cycle, increases genetic instability and reduces apoptosis. In conclusion Alien might be a key factor for prevention of tumor development and progression.

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7. Eidesstattliche Erklärung

Hiermit bestätige ich, Robert Kob,
dass mir die geltende Promotionsordnung der Fakultät bekannt ist;
dass ich die Dissertation selbst angefertigt hat und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben habe;
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Bei meiner Promotion haben mich bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts folgende Personen unterstützt: PD Dr. Christian Melle, Prof. Dr. Ferdinand von Eggeling, Prof. Dr. Stephan Diekmann

Jena, den 7. September 2010

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(Robert Kob)

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